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**THE HORMONES**  
**PHYSIOLOGY, CHEMISTRY AND APPLICATIONS**

**VOLUME II**



# THE HORMONES

PHYSIOLOGY, CHEMISTRY AND APPLICATIONS

*Edited by*

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## VOLUME II



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## Preface to Volume II

It was stated in the first volume that the division of this treatise into two volumes is primarily a matter of convenience. Anyone acquainted with the immense ramifications of the hormones would certainly hesitate at the task of dividing the field into two approximately equal parts on any logical scientific basis. The reader will therefore find that while the bulk of the animal physiology is to be found in the present volume, there are numerous exceptions, and some subjects, like bioassay, may appear in both volumes, though without appreciable overlap.

A work such as the present, in which the sections are contributed by experts, inevitably runs certain risks. The most important of these is the risk of disproportion. The value of a complete book, like that of a course of lectures, depends partly on the placement of the different subjects in association with one another so that they can be viewed together and in perspective, and that the general structure of the whole subject can begin to appear. While some critics have pointed out certain shortcomings of Volume I in this respect, it is hoped that the completion of the work may now prove it to be reasonably well balanced. Another risk is that in the division of topics among authors, some area, or some part of an area, may be omitted altogether. While this is certainly undesirable, it must also be agreed that completeness is a goal impossible to attain, and that the rapid development of the subject which is to be expected in the near future will introduce a much more serious source of incompleteness.

For the rest, the very favorable reception given to the first volume confirms the Editors in their belief that there was need for a complete presentation of the subject, at the research level, at this time. The usefulness of the book for reference should be markedly increased by the very thorough indexes to both volumes compiled by Dr. Martha Sinai. It is hoped that the systematic presentation of so much new knowledge in an authoritative and critical way may make possible a more fundamental understanding of the field and serve to stimulate the planning of new and perhaps more broadly-based research.

The contributions of the authors of course speak for themselves, but the Editors cannot forego expressing their cordial appreciation to these authors for their excellent cooperation in the difficult task of assembling the completed manuscripts.

KENNETH V. THIMANN  
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March, 1950

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# CHAPTER I

## The Physiology of Ovarian Hormones

By GREGORY PINCUS

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### I. Introduction

In discussing the functions of the ovarian hormones, we propose confining ourselves to the recognized and established secretory entities of the female gonad. The two chemically determined active hormones of the ovary are: (a) the estrogenic steroids; estradiol and perhaps estrone and (b) progesterone. Progesterone is a product of luteal tissue and is secreted in major amount when active corpora lutea are present (227). Estradiol has been isolated from ovarian tissue and estrone has been indicated as being present (227). Other steroids having little or no biological activity have been isolated from corpus luteum or ovarian tissue extracts, but they will not be discussed hereafter except as they may play a role in ovarian hormone function. Hormonal activity by the ovary which presumably involves substances other than the estrogens and progestins has been suggested by various experimenters. Hill (139), for example, has demonstrated androgen production by ovaries transplanted to the ear. Indications of sex-modifying secretions from embryonic gonads are numerous.

(*vide infra*), but since these substances have not been chemically identified they will be considered only indirectly. Finally, one hormone of the ovary, the chemical nature of which has not been established, deserves more than passing comment; this is relaxin. Relaxin effects have been ascribed to the steroid hormones, but in recent years it has become clear that ovarian extracts containing this principle have peculiar individual effects (122) and with the recent preparation of extremely potent water soluble extracts (4) final chemical identification does not seem too distant.

To survey the enormous literature on the physiological properties of the ovarian hormones is not possible in a review which is of necessity limited. Comprehensive presentation of experimental data on ovarian physiology are available for developments through 1936 in Allen and Doisy's "Sex and Internal Secretions" (10). Developments since that date will therefore be stressed in this presentation. Furthermore, although steroid ovarian hormones are produced in certain plants (cf. 52,128,227) and exert definite physiological effects in plants (cf. Thimann (293)), we shall reserve our considerations to the higher animals, particularly to mammals. Although both estrogenic and progestational activity have been obtained in extracts of ovaries of the lower vertebrates (e.g. 238,297) their role in the physiological economy of these organisms is, in most instances, not too clear, whereas the overwhelming body of experimental data is derived from experiments with mammals. Accordingly the ensuing accounts of the effects of deficiency of ovarian hormone, its administration to experimental animals, and so on, will refer chiefly to mammalian material. Data on non-mammalian animals will be cited only where it is relevant to specific aspects of ovarian function.

## II. Sex Cycles in the Female

It does not appear necessary to review the cyclical nature of ovarian activity. Full descriptions of estrus and menstrual cycles for numerous species have appeared many times. For reviews of the older literature the reader is referred to Marshall (202) and Parkes (224). More recently Asdell (16) has published a compendium of sex cycle phenomena in numerous mammalian species.

The major pertinent consideration is that all female sex cycles include a follicular phase which precedes a luteal phase. The former is characterized by ovarian follicle growth and maturation, the latter by the development of corpora lutea from ovulated (ruptured) follicles, and their eventual involution. In certain species the follicular phase continues until ovulation is induced by mating (e.g., the rabbit, the cat, the ferret), or, if ovulation is not induced ovarian involution may occur with an accompanying anestrus

state. It need scarcely be stated that in these species release of pituitary gonadotrophic hormone in amount adequate to cause follicle rupture depends upon the nervous stimulation resulting from the orgasm. Recent evidence indicates that the release of the requisite pituitary hormone is accomplished by an adrenergic neurohumor originating in the hypothalamus and acting upon the anterior pituitary (200,201). Most species of mammals appear to ovulate spontaneously and the luteal phase follows automatically with the establishment of corpora lutea. In the rat and mouse, however, the corpora lutea formed will not secrete adequate corpus luteum hormone unless copulation takes place at the estrus preceding the ovulation. In these species the nervous stimulation of coitus leads to anterior pituitary secretion of luteotropic hormone. This appears to be mediated by cholinergic neurohumors (291).

The follicular phase of the estrus and menstrual cycles is characterized by cellular proliferation and growth of the tissues of the oviducts under the influence of ovarian estrogens. The estrus climax of the follicular phase may involve temporarily a degree of tissue destruction (*e.g.*, desquamation of vaginal epithelium, uterine bleeding as in the dog), but this is rapidly followed by further oviducal tissue development stimulated by progestin secretion. The development in practically all species of glandular tissue in the uterine endometrium during the luteal phase has led to the designation of this as the stage of secretion. The involution of the corpora lutea ends the luteal phase, and is characterized by the involution also of ovarian-hormone sustained development in the oviducts. Uterine endometrium involution in primates is so marked at this stage that bleeding occurs from the denuding tissue. This menstrual bleeding is preceded by characteristic vasospasm of endometrial arterioles (27) which deprives the mucosa of blood-borne nutrition.

Breakdown and desquamation of the "starved" tissues then ensues. According to Smith and Smith (283) the vasospasm and ensuing menstruation is accountable to estrogen breakdown products which arise with waning of progesterone secretion. Progesterone, according to the Smiths promotes estrogen metabolism to form products which are not vasoconstricting in effect. The autolyzing menstrual endometrium produces a fibrinolytic toxin which also stimulates anterior pituitary secretion (284); so do the estrogen breakdown products responsible for the uterine arteriole vasospasm. As a result of this stimulation, the follicular phase is instituted again.

This unique and interesting theory of the menstrual cycle has not been applied to estrus cycles in non-primates. We shall return to various pertinent considerations in later sections.

### III. The Effects of Ovariectomy

The classical method for establishing the production of internal secretions by any organ or tissue involves as a first step the excision of the presumed endocrine tissue. In the case of the ovaries this has been practiced for many species of animals and often on a large scale both experimentally and commercially. For accounts of early relevant studies of the effects of ovariectomy the reader is referred to the accounts of Marshall (202), Parkes (224) and Allen *et al.* (10). For the purposes of this presentation we will confine our discussion to the effects of this operation on the reproductive tract during estrus and pregnancy cycles and on those non-generative organs having special relations to ovarian hormone function.

#### A. OVARIECTOMY AND THE ESTRUS CYCLE

Ovariectomy abolishes the estrus and menstrual cycles in mature adult females. We recognize two types of estrus and menstrual cycles: (a) non-luteal cycles and (b) luteal cycles. Neither appear after ovary extirpation. If ovariectomy is performed before puberty the female sex cycles never occur. One extremely interesting exception is observed with mice or rats ovariectomized at or shortly after birth. In these animals late in life estrogen production by the adrenal cortex is sufficient to stimulate the infantile reproductive tract to nearly normal function (311a, 312). Generally ovariectomy in mammals leads to involution of the fallopian tubes, uteri, cervices, and vagina. In rodents these plump, highly vascularized organs become thin and anemic. The epithelium lining the lumen of their cavities is reduced to one or two cells in thickness, the muscular layers decrease in size markedly, and the mucosal glands practically disappear. The vaginal smear contains only scant amounts of leucocytes and few epithelial cells.

In primates menstruation is abolished. The phenomena of uterine growth and secretion characteristic of the follicular phase of the cycle do not occur and the secretory endometrium of the luteal phase is absent. Involution of the chief reproductive tract tissues is slow, but progressive. Since a species of ovariectomy occurs in women at the menopause the ultimate atrophy of uterus, cervix, and vagina is an oft-observed natural phenomenon.

Practically all animals, except the primates, lose the capacity to exhibit mating behavior. The effects of ovariectomy on courtship and sexual activity have been recently reviewed by Beach (32).

#### B. OVARIECTOMY AND PREGNANCY

In most species of animals complete ovariectomy during pregnancy is followed by termination of the pregnancy. Those mammals in which abortion does not necessarily supervene after ovariectomy are the guinea

pig (133), the monkey (127), the horse (10) and the human female (15). Presumably placental secretions in these animals are adequate for the maintenancy of pregnancy. Ovariectomy conducted before the implantation of the fertilized ovum not only prevents the implantation but also prevents normal development of the free uterine ova (231). These effects are ascribable to the loss of functional luteal tissue since extirpation of the corpora lutea alone leads to abortion or failure of ovum implantation (61,99,224). In pseudopregnant animals or animals containing pre-implantation ova, ovariectomy or corpus luteum removal hasten the involution of the typical secretory endometrium of the uterus. The uterine growth characteristic of the luteal phase of the cycle is halted and disappears. The vaginal mucosa of rodents loses its mucoid-secreting activity, and the oviducal epithelium generally recedes and becomes much reduced. The uterine and tubal contractions characteristic of the phase of luteal activity also disappear (see Reynolds, 245).

In those species where ovariectomy fails to cause abortion or resorption of embryos the placenta appears to secrete adequate gestation-maintaining hormone. This is particularly obvious in the human female in whom the corpora lutea of early pregnancy involute at about the third month. The presence of pregnancy-maintaining hormone in human placentas has been demonstrated by a number of workers (3,233,281). Even in species in which ovariectomy normally causes abortion the reduction of the conceptuses to a minimum (129) or the retention of one or more fetal placentae with no fetuses (168,221) will result in delivery of the retained fetuses or placentae at term. In these species there appears to be a placental hormone (18) which acts (via the pituitary) to stimulate pregnancy-maintaining hormone from the ovaries.

### C. THE EFFECTS OF OVARIECTOMY ON NON-GENERATIVE ORGANS

Secondary sexual characteristics disappear at varying rates following ovariectomy. The rate of disappearance may be, in part, a function of the amount of estrogen available for circulation in the blood since most of the characteristic female secondary sex traits appear to be due to estrogen action. Certain phases of mammary tissue growth, for example, appear to be directly due to estrogen stimulation (cf. Folley and Malpress, Vol. I) and a degree of mammary involution follows ovariectomy. The lack of marked mammary decline in the postpubertal ovariectomized human female, for example, may be due in part to sustaining estrogen produced by the adrenal cortex, supplemented, perhaps, by dietary estrogen. Similar considerations may apply to the rather slow change in body hair distribution and to the rate of appearance of osteoporosis which characterizes long-time estrogen deprivation (5,6).

Prepuberal castration in female mammals prevents the appearance of various typical secondary sexual characteristics. In the human female, for example, pubic and axillary hair are scant and the feminine distribution is lacking. The pelvic enlargement, and the characteristic fat deposition typical of mammary growth fail to occur. The tendency for generalized fat deposition is observed in most female mammals.

Ovariectomy of cyclic females results in now well-recognized changes in the anterior pituitary. The appearance of the "castration cell" (2) is accompanied by a hypertrophy of the pituitary (93) and an increase in its gonadotrophic potency as determined by transplantation to suitable test recipients (88). The characteristic cyclic degranulation of the pituitary basophiles fails to occur (275). All of the foregoing phenomena suggest failure of discharge of pituitary gonadotrophin at the normal rate with consequent storage of hormone.

The adrenal cortex of mammals also exhibits certain changes after ovariectomy. The hypertrophy reported by several investigators (cf. 173) has not been confirmed by Smith (280) in the rat, who observes decline in sudanophilia and cholesterol accompanied by increased plasma Na and increases in glycogenesis. A release of inhibition of pituitary adrenocorticotropic by ovarian hormone is postulated as the probable causal factor for the increased adrenocortical activity.

Except for possible modifications of thyroid activity discussed in Chapter IV, and reflected in a slight rise in BMR (213) ovariectomy seems to have no demonstrable effect on the activity of the other glands of internal secretion; for example, Haist and Bell (119) found no effect on insulin production of the pancreas; adrenomedullary activity is not affected, nor is pituitary posterior lobe function.

Indications of obvious effects of ovariectomy on other organs and tissues of the body are scant and not too conclusive. Thus the origin of creatinuria following ovariectomy in the guinea pig (58,59) has not been elucidated. It probably does not involve alteration in kidney threshold since no change in either diodrast or inulin clearance occurs after ovariectomy (304). Other somatic functions appear to be undisturbed by ovariectomy, e.g., liver, heart, brain etc. although relative hypoplasia has been observed, and thymus hypertrophy appears invariably (174). A recent discussion of castration obesity is given us by Korenchevsky and Jones (174). A reported decline in brain cholinesterase (162) requires confirmation. Changes in concentration of certain blood constituents have been reported. These include a depression of blood calcium (106,276) especially in birds, a decline in plasma phospholipids (51), a mild increase in red cells and hemoglobin (288), but no other notable alterations. The blood calcium changes are generally associated with estrogen effects on skeletal development. Ovariectomy in mammals generally leads to an immediate

stimulus especially to long bone growth which may not last into adult life (106). The skin and integumentary system are clear target organs for ovarian hormones and ovariectomy therefore leads to appreciable alterations. The changes in bird plumage have been described in detail by Domm (84). In mammals the effects of ovariectomy are not so striking although the loss of skin "tone" in post-menopausal women has been attributed to estrogen deprivation. Experiments on estrogen administration have been more instructive in this regard (cf. 108,146,217).

#### IV. The Effects of Replacement Therapy with Ovarian Hormones

The administration of ovarian hormones to ovariectomized animals involves essentially the artificial creation of estrous states with estrogens and of progestational states with gestagens. Actually there is a close sequence of interaction between these two major types of ovarian hormone. In this section we will consider, first of all, effects on behavior, then specific tissue responses, and finally certain extragenerative manifestations.

##### A. BEHAVIORAL RESPONSES

The obvious induction by estrogens of morphological evidences of estrus in female castrates (e.g., vaginal cornification, uterine hypertrophy) may not always be accompanied by copulatory behavior. Estrogen replacement therapy alone appears to evoke copulatory responses (7,76,112,222, 223) in fish, reptiles and birds, but among mammals certain and regular evocation with estrogen alone appears to occur only in the cow, the ferret (203), the dog (176,181), the cat (26,198) and the rhesus monkey (24). In the rat, mouse, guinea pig, and hamster irregular and inconsistent responsiveness consequent to estrogen administration is made sharp and more like the normal pattern when small estrogen doses are followed at appropriate intervals by progesterone administration (29,36,37,100,165,256). The species specificity of the progesterone effect is indicated by the ineffectiveness in the guinea pig, at least, of related steroids as substitutes (e.g., pregnanediol, pregnanetriol, androstanedione, testosterone and others (137)), and the reduction by progesterone of estrogen-induced sexual activity of the monkey (25). It is possible that estrogen-induced copulatory behavior in spayed animals may involve steroid hormones, produced (perhaps reflexly by stimulation by the estrogen of adrenocorticotropic hormone release (292)) by the adrenal cortex; this is suggested by the efficacy of an adrenal cortex extract as a substitute for progesterone in the guinea pig reaction (295). Also adrenal production of progesterone is likely (33). Ring (257) has observed practically complete inability of the spayed adrenalectomized rat to exhibit sexual activity after estrogen administration, but effective restoration with the estrogen:progesterone treatment.

The extent of hormonal control of sexual behavior in the ovariectomized

woman is a highly controversial matter. Restoration of libido to such subjects has been claimed for estrogen (132) but androgen has been found equally effective (264). In fact, even in the rat in which most estrous phenomena are clearly hormonally conditioned, testosterone administered to the prepuberally spayed female has led to female-sexual receptivity as well as male-like copulatory behavior (30). Beach (31,32) has discussed in detail the interaction of established neural conditioning and administered sex hormone.

The role of extra-ovarian factors in other hormone-conditioned activities is illustrated by the restoration of cycles of running in spayed female rats receiving a constant supply of estrogen (248,314). Since progesterone did not affect the estrogen induced activity it seems that running and mating cycles have a different conditioning in this species. Data on other behavioral phenomena after ovarian hormone administration to ovariectomized animals are too scant for significant analysis (cf. 32).

#### B. RESPONSES OF THE REPRODUCTIVE TRACT

The vagina, uterus, and fallopian tubes are characteristic target organs for the ovarian hormones. The administration of the active crystalline ovarian steroids effectively prevents their castration atrophy. Recapitulation of follicular phase effects is largely an estrogenic function whereas luteal phase phenomena are optimally attained by the combined action of estrogen and progestin. It is not necessary to review here the early experiments involving replacement therapy which led eventually to the isolation and identification of the active ovarian steroids. The use of spayed animals as essential to assay led to the quick recognition of typical effects. That the familiar stimulation of the quiescent vaginal epithelium to growth, stratification and cornification is a direct effect of administered estrogen is attested to by vaginal application. As little as  $25 \times 10^{-5}$  micrograms of estradiol instilled in glycerine solution will cause these typical effects in castrated mice (215) and for true estrogens the minimal effective intravaginal dose varies from 1/50th to 1/2000th of the minimal effective subcutaneous dose (86). Restoration of normal stromal size and tone is also a function of estrogen action, whereas the vaginal mucification characterizing pregnancy appears to require the simultaneously balanced action of estrogen and progestin (12,169), although pregnanediol (101) and androgen (193) may substitute for progesterone. Excess of progesterone not only abolished estrogen-induced cornification (66,274) but also the mucification reaction (169). Although the typical cyclic vaginal changes may be induced by appropriate estrogen and progesterone administration the observation that constant estrogen administration to the rat (38) and of estrogen and progesterone to the chimpanzee (56) is accompanied by cyclic change suggests an extra-ovarian regulation.

The mechanisms involved in these typical vagina-stimulating effects are still obscure. Although the vaginal pH (elevated by ovariectomy) is markedly lowered by estrogen administration (120,267), the relative acidity cannot account for any of these phenomena. Rather the acidity may result from lactic acid derived from the large glycogen deposition that follows estrogen administration (298). The recent observation of Jeener (155) of the increase in alkaline phosphatase and cytoplasmic ribonucleic acid of the spayed mouse vagina following estradiol administration offers a lead to possible biochemical processes underlying vaginal responsivity. The enhanced vaginal response to estrogen of spayed alloxan diabetic rats vaguely suggests a "carbohydrate" factor in the responsivity (278). A comprehensive account of both uterine and vaginal epithelium characteristics in the human has been presented by Papanicolaou *et al.* (223a).

The characteristic uterine responses of ovariectomized animals to ovarian hormones need not be repeated in detail. The initial effect of estrogen administration in practically all species is a hyperemia leading to a rapid accumulation of water which is then followed by the characteristic hypertrophy of both endometrium and myometrium (cf. 48,305a). Adrenalectomy in the spayed rat increases uterine sensitivity to estrogen whereas adrenocorticotrophin administered to the non-adrenalectomized spayed female decreases uterine sensitivity (290). This suggestion of an adrenal "brake" on estrogen action may involve endogenous progesterone or 11-deoxycorticosterone since the administration of these substances but not of carbohydrate active corticosteroids inhibits the uterine water increase following estrogen administration to the spayed rat (290).

The biochemical events mediating the estrogen effect on the uterus are not too well defined. The increase of anaerobic glycolysis in the spayed rat uterus following estrogen administration (166,289) seems to precede in time the proliferative response and the maximum is attained at about the time that maximal glycogen deposition takes place (35). It is interesting to note that the respiratory effect cannot be effected *in vitro*, nor even by direct injection into the uterus just before excision, suggesting that an "activated" estrogen is necessary (289). Similar activation of estradiol by the rat liver is necessary, according to Roberts and Szego (258), to effect the earlier water increase. Alkaline phosphatase also increases significantly in the uterine epithelium and glands following estrogen administration to spayed monkeys and is reduced after progesterone injection (19). The human uterine endometrium exhibits a rise in alkaline phosphatase during the proliferative phase with a decline in the secretory phase (14,19). Glycogen deposition in the primate uterus appears to be dependent on progesterone action (141,210) and certainly characterizes the secretory endometrium (14,278) of the normal cycle. This appears to be the reverse of the situation in the vaginal mucosa (298).

The types of uterine contraction characterizing estrus and pregnancy can be elicited in spayed animals by estrogen and progestin, respectively. This and the hyperemia preceding it have been characterized by an increase in acetylcholine concentration in the uterus (244,245) but acetylcholine mediation of the effect has been questioned since intraocular endometrial transplants in the rabbit exhibit characteristic vascular responses to estrogen (161). Estrogen augments the uterine contractile responses to pituitrin both *in vivo* (260) and *in vitro* (259). *In vivo* effects of estrogen cannot be attributed to reflex stimulation of the posterior pituitary since they may be elicited in the hypophysectomized animal (260). Similar sensitization to the action of ergot (242) and to adrenaline in the dog and cat uterus (73) has been reported. A rhythmic contraction of the vas deferens of castrated male cats is also induced by estrogen and augmented by pitocin, adrenalin and by stimulation of the hypogastric nerve (205). These effects on uterine motility are counteracted by progesterone which acts essentially to reduce violent contraction (245). Progesterone administration effectively prevents the uterine contractions induced in the estrogen-primed castrate uterus by oxytocin, histamine and adrenaline (295a).

In animals spayed shortly after the time of fertilization estrogen impairs the descent of the fertilized ova (305) whereas progesterone stimulates the uterus to provide adequate nutrition for the free ova (231) in addition to stimulating mucosal proliferation essential for nidation (232). The chemical isolation and identification of the corpus luteum hormone, progesterone, was facilitated by an assay involving the classical pseudopregnant proliferation of the rabbit uterus (see *The Hormones*, Vol. I, Chapter IX). Intensive study has been made of this type of proliferation, and it is apparently best initiated and maintainable in the castrate animal by a balanced ratio of estrogen to progesterone (cf. Courrier, 65). The effect in the spayed rabbit is most easily obtained by initial sensitization with estrogen followed by progesterone administration (142). This holds also for decidiomata production in spayed rats (277). Nonetheless characteristic pregestational response may be obtained with adequate large doses of progesterone (269). Progesterone alone will maintain pregnancy in the spayed rabbit (234) but in this instance cooperative action by placental estrogen-like substances may be suspected. Such considerations do not apply to decidioma developed after progesterone administration to spayed rats (228). An excess of estrogen will prevent progesterone-maintained pregestational effects (63,142,175,261), blastocyst growth (235), nidation (71) and late pregnancy (70). The ability of progesterone and progesterone-estrogen combinations to maintain pregestational reactions and pregnancy appears to be independent of the pituitary (190,243,262). A direct effect of progestin on the endometrium is indicated by the measurable effect of

minute amounts of directly applied hormone: 0.0002 microgram acts directly on the ligated mouse uterine horn (145).

The characteristic effects of progesterone on the uterus, once thought to be quite specific may be produced by other steroid substances. Klein and Parkes' (170) original observation that certain androgens (testosterone, methyl testosterone and others) will cause progestational proliferation in the spayed rabbit's uterus has been extended to the rat uterus (195) and to the maintenance of pregnancy (115). Pregnane derivatives other than progesterone have been found effective in pregnancy maintenance including pregnenolone (70) and 11-desoxycorticosterone (64,249). Masson and Selye (192) find side chain addition at carbon 17 confers luteoid activity on feebly luteoid androstane derivates with methyl, ethyl, and ethynil groups being most effective. The finding that 11-dehydroprogesterone is three times as effective as progesterone in the rabbit (206a) may be only a pharmacological curiosity, but since the adrenal can 11-oxygenate a variety of steroids circulated through it (129a) the occurrence of a new effective circulating progestin is suggested. That practically all injected luteoids must be transformed to a direct-acting substance is suggested by the finding that only progesterone acts in physiological dose in the direct intrauterine test (145a).

The uterus of spayed primates responds to ovarian hormones by proliferative activity when estrogen is given and secretory activity when progesterone is administered. The problem of the hormonal control of menstrual bleeding once so puzzling because it can occur from either a proliferative or a secretory endometrium now seems to be on a rather sure footing. The bleeding may occur as a consequence of the withdrawal or marked reduction of administered estrogen (10,321) and/or progesterone (126,140); as long as these hormones are administered in adequate dosage bleeding will not occur. We have mentioned previously the theory of Smith and Smith (283) that the precipitation of bleeding results from the action of estrogen degradation products rather than from simple withdrawal of hormone support of endometrial cells and blood vessels. Thyroidectomy, however, prevents estrogen withdrawal bleeding in ovariectomized monkeys (89) so that the precipitating factor must act in the presence of adequate thyroid hormone.

Our knowledge of the biochemical conditions within the uterus resulting from hormonal maintenance of the various stages of pregnancy is negligible. A suggestion that certain phases of the pregnancy reactions may be controlled by independent processes comes from the differential action of various estrogens on progesterone-maintained ovum growth and pseudopregnant proliferation (232,235). The work of Chang (55) indicating the lack of uterine receptivity to certain stages of ovum development and the

presence even of ovicidal substances in non-receptive uteri suggests profound variations in activity with time in morphologically similar uteri. Endometrial enzymatic and related changes occurring under hormone influence invite active investigation.

### C. RESPONSES IN NON-GENERATIVE SYSTEMS

The administration of estrogen inhibits the characteristic castration cells of the pituitary (220) and the effects seem to be independent of normal anatomic connections since it will occur in intraocular pituitary transplants (204). The wave of pituitary acidophile mitoses consequent to estrogen administration (178) is not affected by thyroidectomy (22). A rather direct effect of ovarian hormone upon the pituitary is suggested. Castration hypertrophy and gonadotrophic hormone release are also markedly influenced by steroid hormones, but this subject will be discussed in detail in Chapter VI.

The familiar hypertrophy of the adrenal cortex caused by estrogen administration (292) involves a stimulation of adrenocorticotrophic hormone production since it will not occur in hypophysectomized animals (273). Progesterone allegedly does not have this adrenal-hypertrophying effect (270), but recent careful measurement indicate it is merely quantitatively less (167). Kimeldorf and Soderwall (167) find that estrogen administered to spayed guinea pigs causes hypertrophy of zona glomerulosa and fasciculata cells whereas progesterone causes enlargement of glomerulosa and reticularis zones. The basis for this interesting difference remains to be established. The fact that progesterone perhaps by virtue of its adrenocorticotropic actions inhibits pituitary adrenocorticotrophin (57,265) adds to the puzzle.

The inhibition of general body growth resulting from estrogen administration to castrates involves suppression of pituitary growth hormone production (241) although there is some direct action of estrogen particularly on bone (116). The effects on the skeletal system of ovarian hormones administered to castrates have been extensively reviewed (6,107). The estrogens appear to be significant agents promoting medullary proliferation of bone, presumably by stimulating osteoblast activity. These effects may be independent of blood calcium changes and apparently also of parathyroid activity since "hyperossification" occurs in parathyroidectomized animals (23). The anabolic effects of estrogen administration to hypoovarian human subjects are accompanied by nitrogen, phosphate and sodium retention, and this is not due merely to a contribution of these substances to the skeleton (171). That it is a specific sex hormone effect seems dubious since testosterone has similar metabolic effects (171) but certainly acts differently from estrogen on numerous end-organs, including the skeleton.

The effects of replacement therapy upon other non-genital systems have received only limited and rather scattered attention. Intensive studies by Korenchevsky and collaborators on the effects on thyroid, thymus, spleen, liver, kidney, heart and body fat of estrogen administered to spayed rats have recently been summarized (112,156). On a fat-free body weight basis the hypoplastic thyroids, liver, kidneys, and heart of the ovariectomized rat are hypertrophied by moderate dosages of estrogen and thymus hypertrophy reduced. Spleen weight which is somewhat reduced by ovariectomy returns to normal when estrogen and thyroid medication are combined. The primary histologic effects of ovariectomy on the kidney and liver are: (a) decrease in number of mitoses in both organs; (b) decrease in number of large nuclei of liver cells and (c) decreased size of renal tubules (156). Restoration to normal is effected by estrogen and a cooperative action with thyroid hormone is observed. The effects of estrogens on ovariectomized fowls have been extensively studied (cf. 84,115a), particularly as regards plumage changes. Their action in bringing about female plumage colors appears to be directly on melanophore differentiation since tissue culture of dorsal skin from embryos with added estrogen led to typical pigment (123). Action of estrogen on the feather germ also is indicated (307).

A physiological similarity of the vulval and nasal mucosa is indicated by the response of the latter to ovariectomy and estrogen replacement therapy. The periodic hyperemia with reddening and swelling of the conchae occurring maximally in the premenstruum is abolished by ovariectomy and restored by estrogen administration (211). Some repair of the involuting mucosa by estrogens has been observed in the castrated rabbit (230) and beneficial effects have been reported in human atrophic rhinitis (212,263a). In this connection it is interesting to note that both oral epithelial smears (316) and smears of urinary sediment (82) closely parallel the vaginal smears in women and show similar cell types in response to estrogen therapy.

## V. The Ovarian Hormones in Females with Intact Gonads

In considering responses to ovarian hormones in females with intact gonads, one must recognize immediately the complications introduced by the presence of these organs. The ability of ovarian hormones to suppress or stimulate pituitary gonadotrophins leads to effects on endogenous hormone secretion which may markedly alter and even reverse the characteristic actions of exogenous hormone in a single instance. A single injection of estradiol into the ovariectomized guinea pig leads to a clear reduction of citric acid excretion which lasts for 24 hours; exactly similar injection into intact females results in no change of citric acid excretion (216). The inhibition of the estrogen effect in the intact female may be due to the

antagonistic action of an ovary-secreted substance. It may be that the administered hormone is destroyed or excreted more rapidly in the intact animals, or the administered estrogen acting on pituitary gonadotrophin secretion may affect differentially endogenous hormone secretion so that an ovarian antagonist, say progesterone, is released. Examples could be multiplied but it should be recognized that we are dealing not merely with quantitative changes in responsiveness; sharp qualitative changes having a rather complex basis may be involved. In selecting, in the ensuing account, the obvious involvements of ovarian hormones in vital processes, a neglect of the less obvious involves no disrespect for reported phenomena, but merely an inability to understand their bases.

#### A. BEHAVIOR RESPONSES

Estrous states may be evoked by estrogen administration to females either during the diestrus (124) the anestrum (196) or in the prepuberal state (98) and in cattle nymphomania may be evoked (313) by continuous administration (e.g., by pellet implantation). Progesterone administration tends to inhibit copulatory responses in the rabbit (199) and the ferret (203) and reputedly (like desoxycorticosterone) causes anaphrodisiac effects in women (113). Fairly direct antagonism is here suggested since sexual activity of the estrogenized spayed monkey is diminished by subsequent progesterone administration (25). The hormone-induced copulatory responses are not abolished by thyroidectomy (97) and hypophysectomy (198a). Aggressive behavior in animals appears to be influenced somewhat by ovarian hormones. Thus estrogen pellet implantation in heifers may lead to marked increase in fighting activity (124). Estrogen-injected capons on the other hand exhibited timidity and lowered aggression (77). Generally androgen administered to either sex heightens aggressive tendencies (32). In human male psychotics, relative excess of endogenous androgen appears to intensify either heterosexual or homosexual aggression, whereas a female androgen:estrogen ratio is found in timid, withdrawn men (148a).

Maternal behavior appears to be conditioned by ovarian hormones since care of young is eliminated by estrogen administration (118,301). Riddle (250) believes that this maternal care is due to prolactin action in animals previously subjected to ovarian hormone action; he and his coworkers have made extensive studies of hormone-induced broodiness in birds and of maternal care in rats (cf. 251,252). Nalbandov (219) attributes the alleged effects of prolactin to depressed steroid hormone production since administered androgen will overcome prolactin-induced broodiness in the cockerel. This is consonant with the continuation of maternal behavior in hypophysectomized mice which have lost lactating activity (183).

### B. RESPONSES OF THE REPRODUCTIVE TRACT

The induction of vaginal estrus in immature female mammals is accomplished by natural and artificial estrogens. Among the natural estrogens estriol has been found by several authors to be especially potent in this regard but relatively weak in the spayed female (e.g., 206). Similarly lumiestrone (see *The Hormones*, Vol. I, Chapter X), ineffective in spayed animals, causes a more prolonged estrus than estrone in immature rats (92). A pituitary stimulating effect is suspected although conversion of these substances by the ovaries to more potent estrogen is not ruled out. Continuous estrogen injection in mature animals may lead to continuous epithelial cornification or its disappearance, due undoubtedly to variable effects on the pituitary (50). Mucification of the immature rodent vaginal epithelium is accomplished by the proper ratio of estrogen and progesterone (83), but the mucous secretion characteristic of heat in the cow may be evoked by estrogen alone (41); in this and other instances the possible participation of adrenal progestin is not excluded. In women, cervical mucous secretion is induced by estrogen (1) administration and is clearest and maximal in normal cycles at about ovulation time (49). As in castrates typical estrogen effects may be abolished by progesterone or androgen administration.

The oviducts of immature animals respond initially to estrogen administration with a mild hyperemia and water imbibition (17). The vasodilation may be due to histamine release, perhaps as a result of histaminase inhibition by estrogen (143). The initial water intake is maximal at 6 hours after injection in rats. There is then a large secondary edema 18 to 40 hours later. In the primate endometrium both estrogen and progesterone cause a vasodilation but the progesterone effect is distinctly less (189). The synergistic effect of estrogen and progesterone may also be elicited in immature females and forms the basis of the Clauber assay for progesterone (*The Hormones*, Vol. I, Chapter IX). The estriic acids appear to be less effective as uterine estrogen effectors than natural estrogen (69). The response of the chick oviduct to administered estrogen is inhibited by folic acid deficiency (134) or by the administration of folic acid antagonist (135). Similar inhibition of the response of the sex skin in monkeys to estrogen has been observed (136). The inhibition is overcome by liver supplement and one is reminded of the "activation" of estrogen by the liver (258).

The abortifacient effect of estrogen, particularly in early pregnancy in most mammals is now commonplace. Estrogens are most effective in preventing nidation, but in late pregnancy larger quantities are needed (117,225).

Indications that progesterone may prevent pregnancy by inhibiting

fertilization are had by the finding that the presence of corpora lutea in the rabbit ovary prevents the fertilization of superovulated ova (218); pretreatment for 10 days of estrous rabbits with progesterone reduced the fertilization of shed ova and the progesterone inhibition could be overcome by estrogen administration during the last 3 days of progesterone treatment (39).

In the human female massive estrogen doses are tolerated, particularly after the first trimester, with no signs of pregnancy interruption (164). Nonetheless expected increase in excretion of pregnanediol fails to occur indicating inhibition of progesterone production (78) probably by depressing the utilization of chorionic gonadotrophin. Since spontaneous abortion in the human female is very frequently preceded by a drop in pregnanediol excretion (154,263 and others, cf. 284) progesterone administration as a prophylactic and therapeutic measure has been advocated and in some instances found successful (121).

Since prolactin is luteogenic this substance has been found useful in preventing habitual abortion (177). For late pregnancy toxemia estrogen and progestin treatment have been disappointing therapeutically and Smith and Smith believe that not replacement therapy but prophylactic treatment with a gonadotrophin stimulant is preferable (284), since the latter sustains placental secretory activity. Since stilbestrol appears, on the basis of animal experiment (282), to be a notable stimulator of gonadotrophin output its prophylactic administration has been undertaken with marked ensuing amelioration of pregnancy complications and considerable antiabortive effect (285). Bradbury, Brown, and Gay (30a) find chorionic gonadotrophin to be a more active lutecogen than ICSH (see also 50a).

Progesterone administered through the time of expected parturition will prolong pregnancy (130,237). The production of a new set of corpora lutea by gonadotrophin injection on the 25th day will extend pregnancy in the rabbit to forty days instead of to the expected 32 days (287). The inhibition of labor may be due to progesterone inhibition of oxytocin action on the uterus.

### C. RESPONSES OF EXTRA-GENITAL SYSTEMS

The effects of ovarian hormones on pituitary and adrenal function are discussed elsewhere in this volume (Chapters V and III). In intact mammals the adrenal hypertrophy effect of estrogen (8) appears to be due to the stimulation of pituitary adrenocorticotrophin (ACTH) output whereas the atrophy following progesterone and 11-desoxycorticosterone administration (271) is accountable to inhibition of ACTH secretion (265). It is interesting to note that the estrogenic substances most effective in stimulating ACTH output are also excellent stimulants of pituitary ICSH output (282,286).

Ovarian hormone effects upon the skeletal system are not appreciably different in intact animals from those observed in ovariectomized animals. In birds the effects are most striking; the prompt increase in plasma calcium and phosphorous accompanying hyperossification in pigeons described by Riddle and his associates (253,254) and observed also in estrogen-treated chicks (96) does not occur in the rat (43), although a belated hypercalcemia has been observed in the guinea pig (236). The functional activity of the increased blood phosphorus has been demonstrated by the finding that radiophosphorus is incorporated into pigeon bone after estrogen administration (111). In mammals estrogen inhibits bone resorption (279), the rate of healing of fibula fractures (42) and stimulates endostial bone formation (6,107). Bone marrow and gingival damage has been observed especially in dogs (317). It is interesting that the estrogen-induced hypercalcemia of birds is paralleled by a lipemia which fails to occur in mammals (255). Progesterone is relatively ineffective by itself nor does it alter the estrogen effects (255).

That the skin and associated systems are affected directly by estrogenic substance has been established in large part by experiments with topical application (cf. 287a). Thus Selye (272) observed a notable cutaneous edema after stilbestrol inunction in the genetically hairless mouse. Both mammal nipple growth and pigmentation are increased by estrogen inunction (287a). The chick comb growth response to androgen is, interestingly, inhibited by simultaneous inunction with estrogen or progesterone (214), but the androgen-induced pigmentation of the sparrow's bill is not inhibited by the ovarian hormones (229). Hair growth in rats is inhibited by estrogen injected at levels inhibitory to general growth (319) and the regeneration of hair is inhibited by topical application (306). Diminution of activity of skin sebaceous glands also occurs after estrogen injection (147) but they are stimulated to increased activity by testosterone (85). The inflammatory and degenerated salivary glands of avitaminotic rats are restored to normal by estrogen administration (160) but the submaxillary gland atrophy found in hypophysectomized rats is abolished by androgen (or pregnant mare's serum) but not by estrogen administration (53). Anal scent glands in the rabbit react equally to estrogen and androgen (62).

Extensive studies of estrogen-induced abdominal fibromas (188) in the guinea pig have been made by Lipschütz and his coworkers. The estrogen effect is limited by androgen, progestin and various adrenal steroids (187). Attempts at clinical application of the antifibromatogenic action of progestin particularly to uterine fibroids have been made (109). The roles of the ovarian hormones in carcinogenesis and anticarcinogenesis generally have been extensively reviewed (11,48,105). The rather widespread carcinogenic action of estrogens upon typical target organs, e.g., mammary glands, uterus and cervix, pituitary and lymphoid tissue (cf. 105) suggest

that excessive stimulation leads to neoplastic growth. Since, however, gestagens and androgens tend to inhibit the carcinogenic estrogen effects and are themselves apparently not carcinogenic to their target organs a more specific role of the estrogens seems to be involved. The anticarcinogenic effects of very large doses of estrogen in experimental animals and the inhibition of certain types of human cancer by estrogen administration (as well as by androgen) further complicates any attempt at a simple concept. The suspicion that abnormal estrogen metabolites might be produced in certain functional states, states in themselves hormonally conditioned, offers an attractive field for research (cf. discussion on 3-desoxy-equilenine in Chapter X of Vol. 1). The clear demonstration by Bittner and his coworkers (34) of the interrelation of genetic factors, milk virus, and estrogens in the genesis of mammary cancer indicates possible modes of conditioning of estrogen carcinogenesis in other organs and tissues.

The anemia induced by estrogen in female dogs (296) appears to be due to bone marrow cell destruction (72,191). The white cell producing elements are first affected then the hematopoietic cells (72). In rats physiological doses of estrogen increase the erythrocyte count, large doses reduce it as well as the number of leucocytes and platelets (91); since the estrogen effects are less pronounced in spayed rats an ovarian inhibitor or a pituitary factor (cf. 95) is suspected.

The influence of the ovarian hormones on various vascular phenomena has been the subject of much investigation. The peripheral vasodilation caused by estrogen has been measured in rabbits and human subjects by Reynolds (243). Improvement of peripheral-vascular disease in most of a large series of patients is claimed for estrogen therapy (194). The alleged hypotensive effects of estrogen (186) have been investigated experimentally in rats with a clear rise in pressure observed in intact animals but a decrease in hypophysectomized animals (182). The involvement of a pituitary factor is obvious and the possible role of induced adrenal secretion requires examination (cf. 60). Induction of a lasting hypertension in female rats by estrogen administration could not be accomplished in 24 days of injection (138). It is not known if the effects of ovarian hormones on vascular systems mediate the clear body temperature lowering effected by estrogen and the raising effected by progesterone (28,294).

The role of the ovarian hormones in observed effects on blood and tissue enzymes is difficult to elucidate. Effects observed *in vivo* may represent rather remote by-products or more direct actions. These effects are estrogen-induced increase in liver cholinesterase (315) and decrease in blood acid phosphatase (43), or the appearance of histaminase in human pregnancy blood (163) as well as association of blood diastase maxima with the luteal phase of the normal human cycle and the peak of pregnanediol excretion in pregnancy (266).

Uterine  $\beta$ -glucuronidase increases on estrogen administration and the effect (unlike other uterine stimulations) is not antagonized by androgen; Fishman believes that the formation of "activated" estrogen may be involved (94).

Studies *in vitro* are still quite scattered but the inhibition by estrogen of liver malic dehydrogenase and the succinoxidase of adrenals, brain, pituitary and ovaries (197) certainly merits further elaboration since direct participation of the hormone is obvious. The parallelism between the glucose oxidation of the brain *in vitro* and the anesthetic effect of various steroids also is very suggestive, especially since the brain succinoxidase activity is not correlated with the anesthetic effect (110).

## VI. Effects of Ovarian Hormones on Embryogenesis

Experimental studies of the role of sex hormones in embryogenesis have been concerned chiefly with two problems: (a) the control of differentiation of the accessory organs of the reproductive tract and (b) the differentiation and development of the gonads. Basic to the understanding and interpretation of the experimental data is the problem, unfortunately not yet solved, of the identity of the effective substances acting on embryonic gonads and the nature of the substances produced by the embryonic gonads. In a superlative review of the subject, Jost (159) points out that the mere demonstration of characteristic effects of steroid hormones upon embryo reproductive tract structures does not prove that the endogenous effector substances are steroidial. Nonetheless the great mass of evidence appears to indicate that substances very much akin to active steroidal hormones are effective both in gonad and accessory structure differentiation.

Before considering endocrine control of the differentiation of embryonic sexual structures it should be stated that this differentiation does not appear to be affected by activity of embryonic pituitary, adrenal or thyroid. Hypophysectomy in embryo amphibia, birds and mammals (102,150,157) does not alter the course of sex differentiation and at most is followed by some diminution in size of sex structures. Although gonadotrophin administration to mammals late in pregnancy may cause some stimulation of embryo gonads (13,303), these may indicate merely responses of infantile gonads and certainly are irrelevant to the consideration of differentiation. Adrenalectomy in the fetus has no effect on mammal sex structures (158, 303), nor does thyroidectomy in other vertebrates (20,148).

Gonad differentiation is conditioned by the action of inductor substances. On the basis of available evidence, Witschi (308) has postulated two such embryo-produced substances, corticin which induces ovarian structure differentiation and medullarin which induces testis structure differentiation. That these morphogenetic substances are produced by

embryo gonads has been demonstrated by the effects of gonad transplants into embryos before or during the stage of sex differentiation. In amphibian embryos in which one gonad is genetically male and the other female, partial transformation of the graft to ovotestis occurs (151). The sperm of the reversed ovarian tissue are fertile and yield expected sex ratios (152). Feminizing action of ovarian grafts on genetic testes has been demonstrated in the chick (311) and masculinizing action of a rabbit embryo testis graft (159). Similarly, parabiotic union of amphibian larvae during the stage of the indifferent gonad leads to clear modifications in genetically heterosexual combinations. Ordinarily the male partner is dominant, but transformation of testes into ovaries is accomplished particularly when a female partner of one species is united with a small male partner of another species (44). Thus the indications are that embryonic testes produce testogenic and embryonic ovaries ovariogenic substances. In amphibia, exogenous androgenic steroids administered at the proper stage of embryonic development may induce development of testes from indifferent gonads (103,207), and estrogenic steroids induce ovary development (239,307). In the fowl, estrogen administered into the egg causes feminization of genetically male gonads, but androgen does not masculinize female gonads, in fact both androsterone and dehydroandrosterone exert feminizing action and testosterone tends to cause regression of the embryonic ovary (307) with one exception (74). Administration of various steroids to mammals has led to no sex inversion (158), although some retardation of development of the heterosexual gonads is indicated (cf. Figs. 1 and 2). As Jost (159) points out it is not possible to state that the embryonic inductors are different from or the same as the familiar sex hormones even though hormonal activity has been extracted from embryo gonads (184,247).

Castration of embryos in triton (80) or the rabbit (158) lead to alterations of development of the accessory organs if the embryo gonads are removed before morphological differentiation takes place. Similar excision of the opossum gonads does not alter in any way the course of differentiation of the gonaducts and related structures (45,208). Jost (159) suggests a functional elaboration of potent embryo hormone may precede morphological differentiation of the gonads in this species.

The administration of sex hormones to embryos leads to diverse effects but generally Müllerian duct structures are stimulated to develop by estrogens and Wolffian duct structures by androgens (Fig. 1). But estrogens may stimulate Wolffian duct development as shown in Fig. 2 (103,114) and androgens may cause Müllerian duct hypertrophy (125). These paradoxical effects indicate either an interaction of exogenous hormone with endogenous mechanisms or that the administered steroid is "ambisexual" in effect in much the same way that certain steroids are both androgenic and estrogenic in adults. Combinations of estrogens and androgens lead

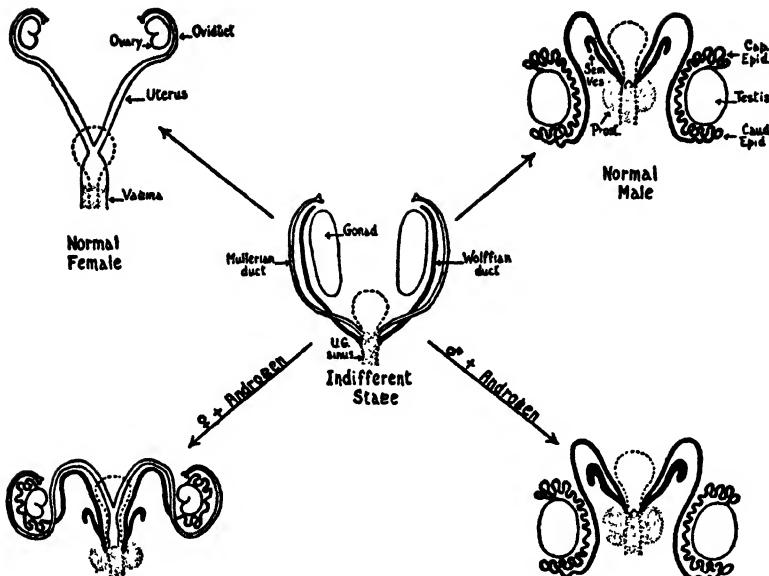


FIG. 1. Diagrammatic representation of normal development of the reproductive tract of male and female rat embryos and the effect when androgen is administered to the mother (from Greene, *Biological Symposia*, 1942).

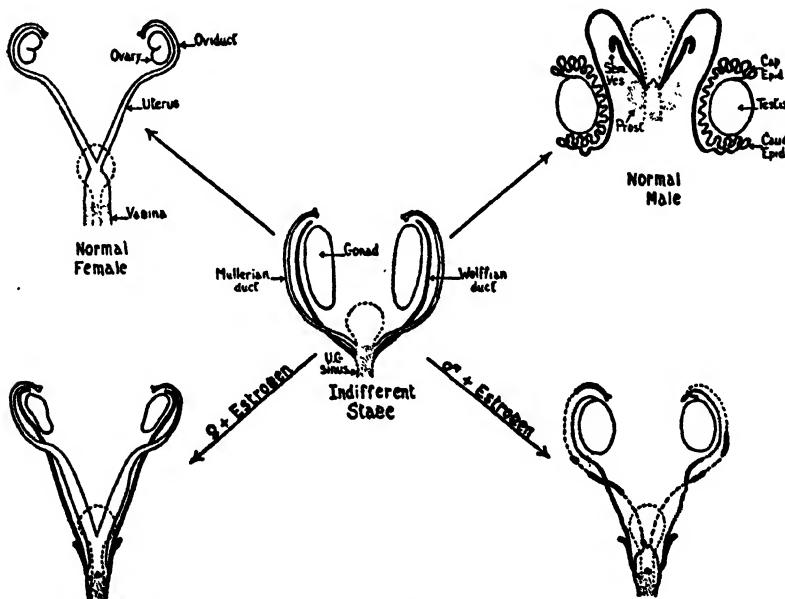


FIG. 2. Diagrammatic representation as in Fig. 1, but showing the effects of estrogen.

to inhibitions of the characteristic actions of each (114). The effects on embryo structures of estrogens administered to pregnant mammals leads to the questioning of lack of such effects in normal pregnancies in which large amounts of such hormone are endogenously produced (e.g., in the horse and the human). Since both estrogen and androgen are present in the maternal circulation there may be a balancing of their respective amounts but more likely embryo sex differentiation is completed well before the large increase in estrogen occurs. Furthermore the need of relatively large amounts of exogenous estrogen to affect sex differentiation in mammals has been noted; androgen, on the other hand, in comparison with effective adult doses is required in relatively small amount (114). It is therefore interesting to note that pregnant women with arrhenoblastomas secreting sufficient androgen to have virilizing effects on the mother give birth to female young having pseudohermaphroditic characters (40).

### VII. Effects of Ovarian Hormones in Males

This subject has recently been reviewed by Emmens and Parkes (87). The chief genital target organs for estrogens in the male are the seminal vesicles, the prostate, and the uterus masculinis. Reactions of extra-genital organs are similar for the most part to those observed in females. Exceptions are found in those organs which, having completed their development as masculine structures, have lost a lability of response present during early development. For example, continuous injections of estrogen begun before epiphyseal closure takes place will maintain open epiphyses for a long time (90). Similarly dwarf rats result from estrogen administration begun at 4 to 6 weeks and continued for several months (318,319), but injections begun after full growth is obtained do not lead to marked loss of weight. This stunting of growth illustrates also the inhibitory effect of estrogen on the secretion of pituitary growth hormone, and calls attention to the distinction between direct and indirect effects. As indicated previously, the estrogens will affect skeletal development directly. The dissociation of skeletal and pituitary-controlled effects has been demonstrated in hypophysectomized male rats receiving pituitary growth hormone and estrogen simultaneously. Adequate doses of the former will induce normal weight gains but inhibition of tail growth (i.e., bone growth) occurred nonetheless (116). In addition to pituitary growth hormone inhibition exogenous estrogen in males affects gonadotrophin, thyrotrophin, and adrenotrophin production. The diabetogenic action of estrogen appears also to be attributable in part to pituitary stimulation and in part to a decrease in the effectiveness of insulin (153). Similarly attributable to gonadotrophin stimulation is the action of estrogen in inducing Leydig cell tumors particularly in estrogenized mice of certain strains (47,144,146).

The direct action of estrogens upon the seminal vesicles and prostates may be demonstrated in castrate male animals. Estrogen in such animals exerts both myotrophic and fibrotrophic effects but little effect on the epithelium in rats (75,172), monkeys (299,323), apes (68), and the ground squirrel (303). Continuous estrogen administration to mice results in striking metaplasia and cornification of the epithelium of the posterior prostatic lobes (46,179) and similar effects in various accessory organs of the reproductive tract follow estrogenization of sexually inactive ground squirrels (302). Cornification of the epithelium of the uterus masculinus occurs after estrogen administration to monkeys (226,299), but the epithelium of the rabbit uterus masculinus is developed by androgen, whereas estrogen stimulates the fibro-connective tissue (87). Male kittens subjected to estrogen administration show epithelial cornification in the bladder as well as notable muscular hypertrophy (239), and urethral cornification has been described in human subjects (209). The embryological bases for the observed responses have been discussed in detail by Zuckerman (322). The mammary glands of male animals are also target organs for ovarian hormones; this is most obvious in the guinea pigs where estrogen alone causes development to the point where prolactin action may cause lactation. Other male mammals show varying degrees of responsiveness (cf. 87). It is probable also that the action of ovarian hormone on the sex skin of male monkeys is a direct effect. Edema of the external genitalia has been described in estrogenized macaques (21), Barbary apes (67) and even in newborn rhesus monkeys (325).

Estrogen administration reduces the volume of seminal fluid in dogs (149) and men (131), but diethylstilbestrol pellets in rams led, at least initially, to an increase in sperm production (54). Pituitary inhibition and stimulation are probably involved.

The direct effects of administered estrogen may be counteracted by adequate androgen administration. These involve not only the seminal vesicles and prostate (81,300,309) but also skeletal changes (106), effects on the uterus masculinus (180,324) and even the effects of the action of estrogen on the male hypophysis (268,310). Emmens and Parkes (87) consider these inhibitions to be due to a direct antagonistic action in the tissues.

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**CHAPTER II**  
**Physiology of Androgens**  
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## I. Introduction and Historical

The problem of the androgens, or the male sex hormones, has developed from a study of a simple relationship of a gland and masculinity to a set of many relationships and interrelationships involving such diverse factors as maintenance of secondary sex characters, general metabolism, and psychiatric relationships. The understanding of these relationships has increased materially with the advances made in the past 15 years, during which period the chemistry of the androgenic hormones has been elucidated and adequate amounts of pure hormones have been made available for intensive experimental work.

The early understanding of the importance of the testis to the male organism comes from the observations made on hypogonadal men. The first empirical observations came from the obvious observations that men without testes showed certain characteristic differences from normal men. The high-pitched voice, the fine texture of skin, and the submissiveness of the eunuch have been known for centuries. The removal of the testes was actually practiced for many centuries for specific reasons. Until the accession of Pope Leo XIII in the nineteenth century, the papal choir in Rome made use of castrated boys to maintain the soprano voices. Men servants were employed who had their testes removed to make them more reliable in the household. Castration was also used as a means of breaking the spirit of men kept in bondage. This practice was known to exist as late as 15 to 20 years ago in some parts of Africa, and may well be practiced today. From these early observations, three relations were indicated, namely, the testis and the pitch of the voice, the testis and the sexual drive, and, finally, the testis and aggressiveness. It remained for modern investigations using careful scientific methods to describe adequately these relationships and to extend them.

One of the earliest workers to describe and demonstrate some of the specific functions of the testis was John Hunter in 1792, who showed that the functional activity of such structures as the seminal vesicles and prostate was dependent upon the testis. The importance of these studies was the fact that Hunter departed from gross observations to more specific observations. This investigation started to accumulate evidence which was to be used a century later in the formation of a working hypothesis of an endocrine system.

Modern work on the androgens began with efforts of Berthold (7) in 1849, who related the shrinkage of the cock's comb after castration to the loss of some material in the testis. By implanting testis into capons this investigator was able to show that the involuted comb could be stimulated to grow. Brown-Séquard (12) claimed to have demonstrated stimulating effects of testicular extracts in experiments conducted on himself. Whether this investigator's conclusions could be accepted, on the basis of his experimental data, is open to some doubt. However, Berthold did demonstrate in a convincing manner that something was produced in the testis which exerted its influence at a distance and apparently independently of the nervous system. This was a finding of fundamental importance. The work of Pézard (143) in 1911, was a further advance. He was able to demonstrate that extracts of testis could actually stimulate the capon's comb. This work was decisive since not only was he able to reverse the castration effects on the comb, but he was able to do this with an extract which contained no viable cells.

The work of other early workers in the field of androgens may be briefly stated. Variot (185) treated patients with testicular extracts and thought that some beneficial effects may have been exerted. Walker (190,191) prepared extracts of cock's testes and found that they caused masculinizing changes when administered to hens, as evidenced by growth of the comb and wattles. Further progress in the study of the androgens awaited the more detailed work of Koch, Moore, Loewe, and Harrow. Essentially, these workers showed that extracts of both testis and urine contained material which could replace certain functions of the testis in both birds and mammals. Descriptions of the isolation of androgens from the testis and adrenal cortex are contained in Volume I, Chapter XII, of this work.

## II. Influence of Androgens on Sex Differentiation of the Embryo

Lillie (112) in a classical study described the masculinizing of a genetic female embryo in the cow when it was twinned with a male. This modified female is known as a freemartin. The male remains unmodified. The freemartin is essentially a sterile intersex exhibiting vas deferens, seminal vesicles, and ovaries containing tubular structures of the seminiferous type. Rudimentary oviducts and uteri are present. One of Lillie's (112) interpretations was that the testes of the male twin secreted androgens which produced the masculinizing effect in the female partner through the common blood supply. Experimental investigations of this effect of androgens has been carried out in a number of species. In this section, representative experiments will be discussed in birds and mammals. An authoritative review of this subject has been published by Moore (128a).

### A. BIRD EMBRYOS

Although attempts were made as early as 1921 by Minoura (127) to evaluate the influence of sex hormones by testicular and ovarian grafts on chick embryos, our knowledge of the influence of sex hormones on embryonic development really dates from the thirties, when concentrated testicular and urinary extracts and pure crystalline hormones became available. It was found later that such early attempts as those of Minoura were not specific sex hormone effects. The first experiments using potent androgenic extracts were carried out by Kozelka and Gallagher (101), who showed that extracts prepared from normal men's urine caused stimulation of the Wolffian ducts of chick embryos and that bull testis extracts had a slight effect on the embryonic ducts. Later pure androgens were studied (198a). The androgens were introduced into the egg between 43 and 72 hours of incubation and the degree of development determined at the sixteenth to eighteenth day. In genetic females, low dosages of androsterone, dehydroisoandrosterone, and testosterone propionate produced enlargement of the right ovary due to hypertrophy of the medullary tissue, and the ovary tended to assume a testislike shape. The left ovary remained normal in form and histology. The Wolffian ducts were affected only slightly, if at all, and the oviducts showed no apparent change. With higher doses of the same androgens the effect is more intense. Both ovaries assume a more testislike form. Hypertrophy of the right ovary as well as the medulla of the left ovary was observed.

In genetic males different effects were observed with different androgens. Testosterone propionate decreased the size of the testis but did not change the structure histologically. Androsterone and dehydroisoandrosterone produced reactions similar in kind to that produced by such estrogens as estrone and estriol. The testes changed to flattened ovarylike bodies with some formation of ovarian medullary tissue, while the oviducts persisted and often hypertrophied. In the genetic males all three androgens produced hypertrophy of the Wolffian ducts and certain mesonephric tubules. Other workers have shown androsterone to have both feminizing and masculinizing effects on the male embryo.

Testosterone propionate has been shown to produce stimulation of the Wolffian ducts and also of the mesonephric elements in male and female embryos (29). When androgens, such as androsterone and dehydroisoandrosterone, are administered before the fourth day of incubation, the development of the Müllerian duct may be completely suppressed.

### B. MAMMALIAN EMBRYOS

The influence of androgens on the developing embryos of such mammals as mice, guinea pigs, rats, and opossum has been studied. Extensive work

on the influence of testosterone, androsterone, dehydroisoandrosterone, and  $\Delta^4$ -androstenedione-3,17 on the rat embryo have been described by Greene, Burrill, and Ivy (46). Genetic females were masculinized, the extent being governed by the length of the injection period during gestation and the quantity of androgen administered. When treatment was started on the fifteenth day or earlier, the degeneration of the Wolffian ducts was inhibited and development of this duct system proceeded to such male structures as epididymis and seminal vesicles. These animals also possessed a vas deferens and ejaculatory duct. The urogenital sinus was completely masculine, consisting of a prostatic complex and Cowper's gland. A typical penis and a penile urethra were found. The upper portion of the female duct system consisted of oviducts, uteri, and upper vagina with the lower portion missing. When androgens were administered after the fifteenth day of gestation, the Wolffian ducts showed more extensive regressions, resulting in less development of masculine structures.

The administration of testosterone propionate to pregnant mice resulted in embryonic changes in the females similar to those found in rats. The degree of masculinity depended upon the duration of hormone administration. The Wolffian duct components were stimulated and the medulla of the ovary showed rete formation with anastomosing tubules which united with an epididymislike structure. These females showed well defined prostates, coagulating glands, and seminal vesicles (148-150,181). Dantchakoff has observed similar effects of testosterone propionate in the guinea pig (29).

### III. Influence of Androgens in Various Animals

#### A. Fish

Although there is no direct evidence for androgens in the tissues or excreta of fish, it is apparent from various studies that androgens may produce profound physiological effects in this group of animals. Other studies involving castration are also of interest with respect to the role of androgens in the physiology of these organisms. Castration has been accomplished in fish, and if the operation preceded the breeding season the development of nuptial characteristics was prevented. Such workers as Kopec (89), Tazawa (178), and Hansen (58) have demonstrated this relationship.

The influence of androgens, particularly testosterone propionate, has been studied in a number of species of fish under various conditions. Androgens administered to either juvenile or adult females produced masculinizing changes. Females of the species *Gambusia holbrooki* Girard treated with testosterone showed changes in external appearances resembling the males, but no detectable changes in the gonads. The anal fin of the female was lengthened to resemble that of the male. In young males

of this species stunting of growth was observed along with acceleration in the development of the secondary sex characteristics (57). The female *Xiphophorus helleri* has been modified toward the male, with respect to dark pigmentation, by testosterone propionate (8). Immature females of *Gambusia affinis* were masculinized as were adult females. The pectoral fins of juvenile males and females of *Gambusia affinis* are practically identical in structure. After sexual maturity a modification occurs in the fin of the male. By treatment of females with methyltestosterone the male type of pectoral fin may be produced (501). *Triton cristatus*, surgically castrated, or in a state of "alimentary castration" produced by withholding all food during the time of sexual activity (July, August, September), responded to androgens with a turgidity of the cloaca, characteristic movements of the tail, growth of crest, and brightening of the body color. The ovipositor of the bitterling has been stimulated by androgens (81). This reaction is not specific for androgens.

#### B. AMPHIBIANS

In amphibians androgens have produced dramatic effects. Again, as in fish, no chemical studies are available with respect to amphibian androgens nor has the normal physiology of such substances been described. However, striking effects of testosterone propionate on metamorphosis has been adequately described and confirmed. Thus when frogs were treated with testosterone propionate either in the larval or tadpole stage all genetic females were transformed into complete males. Larvae of *Rana agilis* and *Rana clamitans* have been so treated (134). The completeness of transformation is evidenced by the production of males capable of producing sperm. Young tadpoles of the indifferentiated race of *Rana temporaria* when treated with androgens were all found to be males. In the adult stage female frogs implanted with pellets of testosterone propionate developed male pigmentation (45). Shapiro (170) has demonstrated that androgens even in the hypophysectomized clawed toad, *Xenopus laevis*, induced ovulation.

#### C. BIRDS

Birds have been subjected to rather extensive research with respect to the influence of androgenic substances on the secondary sex characters. It is well known that, after castration of the cockerel, the comb as well as the wattles regress. The ability to crow is not developed, and the capon never attains the characteristic aggressiveness of the cock. All these involutionary changes may be reversed or prevented by the administration of either extracts of testis or pure androgenic steroids. The females also show certain characteristic changes under the influence of androgens.

The androgenic control of the comb of the common fowl has been studied in some detail. The comb of the male and female chick may be stimulated with androgens (33). This is also true of the capon's comb and the hen's comb. In addition, the wattles may also be stimulated. The histological changes in the capon's comb and the stimulated comb have been described (59).

The beak coloring of some birds is under the influence of androgenic substances. Witschi and collaborators have studied this relationship in some detail. In males of some species the beak becomes highly pigmented during the breeding season, and castration before this time prevents this change. If the male (for example, of the English sparrow) is castrated at a time when the beak is deeply pigmented, the color will fade. The beak of the female does not show the black pigmentation. In the English sparrow the injection of androgenic substances will produce pigmentation of the beak in the castrated male, in the normal male out of season, and in the normal or ovariectomized female (200). Witschi (199,201) has found a similar relationship to exist in the African weaver finch and indigo bunting. In the starling it was found that the androgens are specific for producing a yellow coloration in the beak (202). The crimson color of the beak of the black-headed gull is under the control of the androgens (188).

Androgens may produce changes in the plumage of certain species. In the case of the ovariectomized silver pheasant, testosterone produced feminine plumage (21). In the Seabright bantam the male of the species is hen-feathered and the capon plumage is of the usual male type. Androgens such as  $\Delta^5$ -androstenediol-3 ( $\beta$ ),17 ( $\alpha$ ),  $\Delta^4$ -androstenedione-3,17, testosterone, and dehydroisoandrosterone produce plumage changes in the capon similar to that of estrogens. Androstanediol-3 ( $\alpha$ ),17( $\alpha$ ), however, was ineffective in the dosage employed (31).

The vas deferens of the fowl has been shown to be involuted in the capon and androsterone is capable of restoring the gland to a normal size with normal epithelium (17). Oviduct responses to androgens have been demonstrated in the starling (202), in the night heron (139), and in the dove (151). The oviduct of the adult hen may be stimulated with androgens (202). Androgens produced crowing in the capon along with treading and aggressiveness (156). Male behavior including crowing has been induced in female chicks and hens (54).

In the female canary, testosterone caused suppression of the female reproductive functions, initiation of such male traits as singing, courtship behavior, and peck dominance over normal females, and male type of anal region (172). Androgens maintained spermatogenesis in hypophysectomized pigeons (22).

## D. MAMMALS

1. *Male Sex Organs*

*a. Influence of castration.* If castration is done prepuberally in mammals, the secondary sex characters fail to develop normally. The penis, scrotum, seminal vesicles, and prostate remain infantile. In the rodent the coagulating glands fail to produce sufficient enzyme to cause coagulation of the seminal-vesicle fluid. This phenomenon has been studied particularly in the guinea pig. In the monkey, certain changes in the sexual skin and the nasal mucosa, peculiar to this species, are not found after prepuberal castration.

In man, prepuberal castration produces effects similar to those found for other mammals, but in addition certain other changes are noticed. Thus, the pitch of the voice remains high, the head hairline remains infantile. Pubic and axillary hair are very sparse and facial hair may never develop. Muscular strength may be significantly diminished.

The postpuberal castrate shows changes similar to those seen in the prepuberal castrate with the exception that the effect is less intensive. The physical stigma of castration of both types may be reversed in all mammals with the proper treatment of androgenic material.

*b. Testis.* It has been known for some time that androgens administered to either immature or mature males cause atrophy of the testes. Moore (130) was the first to suggest that this atrophy occurring in the testes is the result of pituitary inhibition or decrease in the production of gonadotrophic hormone. These effects have been demonstrated in rats with testosterone, dehydroisoandrosterone, and androsterone (98,131,132). Studies on guinea pigs have shown that such androgens as androsterone, testosterone, methyltestosterone,  $\Delta^4$ -androstenedione-3,17, and dihydrotestosterone caused atrophy of the immature pig's testis (9,10). That the mechanism is by way of the pituitary has been shown by the fact that no atrophy of the testis occurs when gonadotrophic hormone is administered together with testosterone.

In addition to the indirect effect of androgens on the testes, a direct action of androgens has been established. The testicular atrophy which results from hypophysectomy may in part be prevented by suitable dosages of androgens. In 1933 and 1934 it was demonstrated by Walsh, Cuyler, and McCullagh (193,194) that the administration of the androgen androsterone to hypophysectomized rats prevented the atrophy usually seen. Degeneration of seminal epithelium but not of the interstitial cells was prevented. Thus the reverse of the effect of cryptorchidism is produced when the interstitial cells remain in a normal functioning state while the seminiferous tubules undergo marked degeneration. Nelson and Gallagher (136) were

able to confirm these experiments, and in later experiments Nelson and Merckel (137) extended the work to show that testosterone,  $\Delta^5$ -androstenediol-3 ( $\beta$ ),17( $\alpha$ ), androstanedione-3,17, and dehydroisoandrosterone were effective in maintaining the testes of hypophysectomized rats. In mice a picture quite similar to that of the rat has been shown by Nelson and Merckel (138) after treatment with androsterone, androstendiol-3 ( $\alpha$ ),17( $\alpha$ ), and testosterone. Essentially similar direct effects of androgens on the testis have been shown in the immature and adult ground squirrel (196) during the quiescent state of the animals, in hypophysectomized pigeons (22), and in hypogonadal men (53,174,187).

Recently it has been reported that certain steroids such as  $\Delta^5$ -pregnenol-3 ( $\beta$ )-one-20 and progesterone, which are devoid of androgenic activity, also exhibit spermatogenic activity (1,121).

The maintenance of the seminiferous tubules in hypophysectomized rats is dependent among other factors on the age of the animals at operation and the magnitude of the doses of androgens. When Cutuly *et al.* (27) administered up to 0.3 mg. testosterone propionate per day to rats operated before 29 days of age, no stimulation of the seminiferous tubules could be discerned (26,27). Leathem (106) found the formation of a few spermatids in the seminiferous tubules of rats hypophysectomized at 27 days of age and treated with daily doses of 2.5 mg. testosterone propionate in six of nineteen animals treated from 10 to 20 days. This investigator also found that treatment of animals hypophysectomized when 33 days old with 2.5 mg. testosterone propionate for 5 to 15 days resulted in testes with spermatids and spermatozoa. The cryptorchid testis of the rat, which normally shows involutional changes in the seminiferous tubules, may be maintained with androgens in the absence of the pituitary (56).

c. *Scrotum.* The scrotum decreases and becomes depigmented after either castration or hypophysectomy. Adequate treatment with testosterone propionate either prevents or reverses these changes depending on whether treatment is initiated immediately postoperatively or at some time later (28,52,53,53a).

d. *Penis.* In the castrated rat the penis is distinctly decreased and the administration of androgens such as androsterone, androstanedione-3,17, and  $\Delta^4$ -androstenedione-3,17 produced significant increases in the size (96). The treatment of hypogonadal men with testosterone propionate caused marked changes in the penis. Along with stimulation of growth of the penis, androgens can restore, in part, the erectile ability in certain hypogonads and castrates (55). In cases of overdosage in humans, androgens may produce priapism. After castration, the muscles of the penis rapidly atrophy. The bulbocavernosus, ischiocavernosus, and levator ani muscles atrophy. Androgens prevent or correct this atrophy in whole or in part

depending on when the hormones are administered and the dosage employed (189).

*e. Seminal Vesicles.* Castration or hypophysectomy in the male results in an involution of the seminal vesicles. A variety of androgens such as  $\Delta^4$ -androstenedione-3,17, dehydroisoandrosterone, androsterone, methyltestosterone, and testosterone are effective in preventing or correcting the atrophic changes (10). Androgens exert their effect primarily on the epithelium. The seminal vesicles have been used as an end point for the quantitative determination of androgens (32a,131,132).

*f. Coagulating Gland.* Walker (519) described a coagulating gland in rodents, especially guinea pigs, distinct from the prostate. Some years earlier, it was shown by Camus and Gley (18,19) that in the guinea pig the ejaculate has the property of coagulating soon after discharge. This, they claimed, was due to an enzyme present in a gland near the prostate. Moore and co-workers have studied this phenomenon in guinea pigs and have shown that after castration the amount of ejaculate decreased and the coagulum was less firm or may fail to coagulate. Depending upon the time of administration, androgens will either prevent or correct the failure of coagulation in the castrated guinea pig. The secretions of the coagulating gland, by direct collection, will produce a coagulum when mixed with relatively large amounts of seminal vesicle fluid (32).

*g. Prostate.* Involutionary changes in the prostate occur after castration in the male. This has been shown in the rat (133), guinea pig (43,163), and in men with benign hypertrophy (71). The rate and extent of involution is dependent in part upon the adrenal. This is strikingly illustrated in mice and rats which were castrated between 1 to 3 weeks of age, where maintenance of well differentiated epithelium persisted after operation (65,66,145). After adrenalectomy of the castrated rat, the involution of the epithelium was markedly accelerated (14).

The work of Moore, Gallagher, and Koch (129) showed that the involutionary changes usually found in the prostate after castration may be prevented by the administration of androgens. These original studies were carried out in guinea pigs, using bull testes and urinary extracts. Similar results have been obtained using a variety of androgens and different species of animals including rats (16,96) and monkeys (205). The principal effect of androgens on the prostate gland appears to be ability to stimulate the epithelium, in contrast to the stimulating effect of estrogens which exert their action on the stroma. Under certain conditions, estrogens may enhance the effects of androgens on the prostate, whereas very large doses of estrogens may inhibit the action of androgens. The latter finding has been applied to the treatment of cancer of the prostate, principally by Huggins (69-72).

Androgens appear to stimulate prostatic secretion. Thus, in castrated dogs, decreases in prostatic secretion have been found during the first few days after operation and complete cessation within the first 3 weeks after operation (68,70). Administration of effective doses of androgens will renew the prostatic secretion within the first weeks of treatment. In human hypogonadism, androgens will initiate or increase prostatic secretion (61).

The relation of acid phosphatase of prostatic tissue and androgens has been studied in experimental animals as well as in human subjects. A relatively large concentration of this enzyme has been found in the prostate (47,103). Malignant prostatic tissue as well as prostatic metastases to bone contain high concentrations of acid phosphatase (72) and the administration of estrogens caused sharp decreases while androgens cause increases in enzyme concentrations (69).

*h. Vas Deferens, Epididymis, Cowper's Gland, and Preputial Gland.* Other structures which have been shown to be under the influence of androgens in the male, at least in part, are the vas deferens, the epididymis, Cowper's gland, and the preputial gland. Castration has been shown to cause degenerative changes in the vas deferens, such as decreased secretion, decreased size of the epithelium, and degeneration of the cilia. From the work of Vatna (186) and Moore (128) on rats and Itho and Kon (73) on dogs, we know that these changes may be prevented with androgens. Other experiments of interest with respect to the vas deferens are those of Martins and Valle (124), who showed that testosterone inhibited the contractions of the vas deferens of monkeys *in vitro*.

Cowper's gland and the preputial gland, quite like the prostate and seminal vesicles, undergo the usual atrophy after removal of the testes and may be stimulated by both androgens and estrogens. The stimulation of the preputial gland and Cowper's gland of rats by androgens may be enhanced with estrogens (182).

## 2. Female Sex Organs

Androgens exert specific effects on certain secondary sex characters of the female. They may produce characteristic changes in the ovary, uterus, vagina, oviduct, female prostate, preputial gland, clitoris, and mammary glands.

*a. Ovary.* In mammals gonadotrophic effects of androgens on the ovary, most likely through the pituitary, have been observed in intact guinea pigs and rats. The administration of testosterone and  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\alpha$ ) to immature rats produced both follicular maturation and corpora lutea formation, while the administration of testosterone to guinea pigs resulted in maturation of follicles which persisted as cysts (160). Follicular

maturity and cyst formation as a result of testosterone propionate administration was reported in mice (167,175).

Various workers have been able to show stimulation of corpora lutea under the influence of androgens. Selye (167) has produced this effect in mice while Korenchevsky, Dennison, and Hall (94) and Freed, Greenhill, and Soskin (40) have demonstrated this effect in adult rats. The latter workers have shown that daily doses of the order of 50  $\mu$ g. testosterone propionate for 16 days produced ovarian atrophy while a larger daily dose of 1 mg. produced a larger ovary with stimulated corpora lutea.

With most chronic androgen treatment, ovarian atrophy was observed which is comparable to that produced by hypophysectomy. However, atrophic ovaries produced in this manner are still responsive to chorionic gonadotrophin (169). Testosterone apparently does not produce ovarian atrophy directly but most likely by inhibiting the normal hypophyseal gonadotrophic hormones.

That androgens may produce a direct stimulation of the ovary in the absence of the pituitary is only clear from work on the toad and pigeon. Androgens produced ovulation in the clawed toad (*Xenopus laevis*) in both intact and hypophysectomized animals (170). Methyltestosterone, testosterone, dehydroisoandrosterone,  $\Delta^4$ -androstenedione-3,17, and androstanediol-3( $\alpha$ ),17( $\alpha$ ) produced this reaction. It is also of interest that ovulation can be induced in the excised ovary suspended in Ringer solution by the addition of testosterone and  $\Delta^4$ -androstenedione-3,17 (171). In hypophysectomized female pigeons, even after a year from the time of operation, the ovaries showed follicular stimulation after treatment with testosterone propionate. Estrone failed to produce similar follicular stimulation (23). Leathem and Starkey (107) were unable to produce a gonadotrophic action on the normal immature ovary by a single administration of testosterone propionate.

b. *Vagina*. The influence of androgens on the vaginal introitus of immature intact rats was reported by Butenandt and Kudsuz (15). Premature opening of the vagina could be produced with testosterone,  $\Delta^4$ -androstenedione-3,17, and dehydroisoandrosterone. Androsterone at comparable dosage levels was inactive, while  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\alpha$ ) was active (159). In addition to premature opening of the vagina, cornification of the vaginal epithelium was observed.

In the adult spayed rat  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\alpha$ ),  $\Delta^4$ -androstenedione-3,17, methyltestosterone, and dehydroisoandrosterone prevent atrophic changes in both the epithelium and stroma of the vagina (97). Thus, androgens stimulate the vagina directly. Actually androgenic stimulation of the vagina can occur in the ovariectomized, the hypophysectomized, and in the ovariectomized and hypophysectomized animal (134).

Such androgens as androstanediol-3( $\alpha$ ),17( $\alpha$ ), testosterone,  $\Delta^4$ -androstenedione-3,17,  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\alpha$ ), dehydroisoandersterone, and androsterone have been shown to produce a progesteronelike response, mucification, on the rodent vagina when administered in combinations with estrogens (120,136).

Testosterone propionate produces atrophic vaginal smears in normal women. In an illustrative experiment (19a) the hormone was administered for a period of 31 days beginning with the twelfth day of the cycle. The dose varied from 25 to 75 mg. every other day. Menstruation occurred 10 days after the first dose on the twenty-second day of the cycle. By the eleventh day of the second cycle a typical atrophic vaginal smear was observed. When the injection of testosterone propionate was stopped the vaginal smear gradually returned to normal. The question arises whether this inhibitory effect is due to diminished production of the estrogens by the ovaries or whether the androgen directly inhibited the estrogenic action on the vaginal tissue. When menopausal castrates were injected with a dose of estrogens (estradiol benzoate) sufficient to produce a cornified vaginal smear, along with fifty times its weight of androgen (testosterone propionate), the estrogenic action was completely inhibited. In the same report, a dosage level of 25 mg. testosterone propionate was found to have no influence on the vaginal epithelium of menopausal subjects (19a).

Estrogenic vaginal smears due to testosterone propionate and methyl-testosterone have been reported in a surgical castrate (158), and in menopausal women (8a,45a,134a). In other studies, testosterone propionate has been reported to be without effect on the vaginal epithelium in menopausal women, in fact it has been claimed that hormone causes actual regression (63a,155).

c. *Uterus.* Androgens produce diverse effects upon the uterus such as an influence on motility, stimulation of the stroma and epithelium, and, under certain conditions, specific progesteronelike effects. In monkeys and humans, the effect of androgens on the menstrual cycle and uterine bleeding has been studied. The uterine stimulation in immature rats is not dependent upon either the ovaries or the pituitary (3,134,157). In ovariectomized rats and guinea pigs, testosterone has been found to cause glandular hyperplasia of the endometrium as well as hypertrophy of the uterine stroma.

Androgens in combination with small doses of estrogens may produce typical progesteronelike effects on the endometrium of the uterus. Thus Parkes and coworkers have shown that, in the intact immature rabbit, testosterone, methyltestosterone, methyldihydrotestosterone, methyl-androstanediol-3( $\alpha$ ),17( $\alpha$ ), and methyl-*trans*-androstenediol may produce various degrees of progestational activity (34). These progestational

effects of androgens may be demonstrated in spayed rabbits. In this connection it is interesting to note that the substitution of an ethynyl group for the methyl group at carbon 17 in methyltestosterone produces a substance of high progestational potency with practically complete loss of androgenic potency. Testosterone propionate may produce a direct progestational response in the endometrium. Mazer and Mazer (125) produced such changes in the adult spayed rat while Noble (140) demonstrated direct progestational effects in the hypophysectomized-ovariectomized rat.

Spayed rabbits treated with estrogens followed by androgens will not show the usual uterine contractions in response to oxytocin or pituitrin (153). This motility-inhibitory action of androgens is similar to that found for progesterone.

Androgens suppress menstruation in rhesus monkeys and humans. Zuckerman (204) has been able to suppress the menses of the intact rhesus monkey by doses of 50 mg. testosterone propionate/week, while Papanicolaou, Ripley, and Shorr (142) were able to prevent menstruation in normal women with the same androgen. Hartman (60) has shown that the menses in monkeys may be delayed by testosterone. In the work of Zuckerman, testosterone inhibited both follicle stimulation and luteinization which resulted in suppression of the menses.

Testosterone propionate has been used for the control of functional bleeding. In a series of fourteen such cases, Loeser (115) reported improvement after androgen therapy. In another study by Geist, Salmon and Gaines (42), of 21 patients with abnormal uterine bleeding but with no evidence of organic pelvic disease, all but two showed improvement with androgen treatment. The amounts administered, which varied from 300 to 1000 mg./month, inhibited menstruation and arrested the development of the endometrium at the early proliferative phase. With larger doses, hypoplasia or atrophy could be produced. The effect was apparently produced by inhibiting the gonadotrophic factors of the pituitary. The influence of androgens on menstruation is dependent on the time of administration. When relatively large doses of testosterone propionate (500 to 1000 mg.) are administered during the first 10 or 12 days of the cycle, menstruation may be delayed for as long as 4 weeks. Regressive changes are also found in the endometrium. However, if similar doses are employed during the later part of the cycle, no change in the next expected cycle is found (160,164).

Studies by Engle and Smith (35) and Hisaw indicated that testosterone has a weak progestational action on the reproductive tract of both the castrated and intact immature female monkey. This androgen when administered in adequate dosage will show the following progesteronelike

actions: It will inhibit estrogen withdrawal bleeding as well as precipitate bleeding in the presence of a maintenance dose of estrogen. It will greatly decrease cornification of the vaginal mucosa and inhibit the estrogenic action on the cervix. The edema of the sexual skin caused by estrogen will be decreased by testosterone.

*d. Preputial Gland.* Quite analogous to the effect of the androgens on the preputial glands of the male is the effect on this gland in the female. In the spayed rat, testosterone,  $\Delta^5$ -androstenediol-3( $\beta$ ), $17(\alpha)$ , dehydroisoandrosterone, and androsterone have been shown to cause enlargement and development of the female preputial gland (93,125,182). Of interest is the fact that androgens may not only restore the preputial glands to their normal size in spayed females, but may actually produce glands many times the normal size of those in intact animals.

*e. Vestigial Prostate.* The vestigial prostates of female rats either in the intact or spayed animal may be stimulated by various androgens. The effective androgens include testosterone, androsterone, dehydroisoandrosterone, androstandenediol-3( $\alpha$ ), $17(\alpha)$ ,  $\Delta^5$ -androstenediol-3( $\beta$ ), $17(\alpha)$ , and  $\Delta^4$ -androstenedione-3, 17 (92).

*f. Clitoris.* As early as 1916 Steinach described the growth and development of the clitoris to a penislike structure in spayed guinea pigs which carried testicular grafts. Other workers were able to produce similar effects in various other species. Thus Romeis (154) produced a penislike structure in the dog while Sand (161,162) produced the condition in rats as well as in guinea pigs. With the advent of crystalline androgens, it was shown that the clitoris does, in fact, respond to androgens. This effect was produced in the rat, guinea pig (30), ape (204), and women (160,172a). Testosterone, androsterone,  $\Delta^4$ -androstenedione-3, 17, and  $\Delta^5$ -androstenediol-3( $\beta$ ), $17(\alpha)$  have been used to produce stimulation of the clitoris.

Clinically, growth of the clitoris either in the immature or adult female has been repeatedly observed in cases of adrenal tumors. Here it is usually demonstrated that the body fluids contain an abnormally high concentration of androgens.

*g. Urethra.* Androgens have been shown to be effective in producing a distinct effect on the female urethra, and in rodents a condition of hypospadias may be produced by high doses. In the early experiments of Lipschütz (114), this condition was produced in the spayed guinea pig after the transplantation of testes. Other workers have been able to demonstrate this phenomenon in the young of animals treated with androgens during pregnancy (46,49). Administration of androgens within the first 2 weeks of life in female rats has resulted in hypospadias.

*h. Mammary Glands.* Androgens have an inhibitory action on lactation but under specific conditions a stimulating effect on the nipple, the mam-

mary ducts, the acini, and the alveoli. In both rodents and humans, it is possible to suppress lactation with androgens, particularly with testosterone propionate. Robson (152) and Folley and Kon (39) have studied this response in mice and rats, respectively. A daily dose of 0.1 mg. testosterone propionate suppressed lactation in the mouse, whereas in the rat a daily dose of 0.4 mg./100 g. body weight was used.

Concerning the question of how androgens suppress lactation, the results of Meites and Turner (126) are of particular interest. They were able to suppress lactation in the first 6 post-partum days in rats at which time the prolactin concentration of the pituitary remained unchanged.

Testosterone propionate when administered to lactating females in adequate amounts and over a long enough time causes cessation of lactation despite the stimulation of suckling (67). In post partum women, a total dosage of from 30 to 100 mg. testosterone propionate, given over a period of 24 to 48 hours, is usually effective in suppressing lactation (5,102,144). Doses of 125 to 150 mg. testosterone propionate were used in a series of fifty cases. Effective relief from painful congestion of the breasts was reported in 47 of the cases (173).

Androgens produce a direct action on the nipple. Thus, in the spayed adult rat Noble (140) was able to produce distinct hypertrophy of the nipples and mammae with testosterone propionate. Even after hypophysectomy and spaying, testosterone propionate caused distinct hypertrophy of the nipples but the mammae became atrophic. From the work of Bottomley and Folley (9,11) and Jadassohn, Uhlinger, and Margot (74) we know that the nipples of the guinea pig may be stimulated with androgens and normally this effect is at least in part under control of the testis. The former workers were able to show that castration of the male guinea pig causes cessation of growth of the nipples. Androgens such as testosterone, dehydroisoandrosterone, 17-methyltestosterone,  $\Delta^5$ -androstenediol-3( $\beta$ ), 17( $\alpha$ ) androsterone,  $\Delta^4$ -androstenedione-3,17, and androstanedione-3,17 appear to be active. The latter workers reported nipple stimulation with adrenosterone and corticosterone in addition to estrogens.

Growth of the mammary ducts and acini takes place under the influence of androgens. Nelson and Gallagher (136) reported the complete development of ducts with some acini after the administration of androstanediol-3( $\alpha$ ),17( $\alpha$ ) to spayed rats over a 30-day period. In other experiments, a much smaller but yet significant effect was noted in mice (183), guinea pigs (11), and monkeys (38).

In a detailed study on monkeys, Van Wagenen and Folley (184a) showed that treatment with testosterone in the preadolescent ovariectomized female rhesus monkey induced a secretory state in the mammary-gland epithelium in those alveoli which already existed. It was further observed that de-

hydroisoandrosterone and  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\alpha$ ) caused some secretion and duct dilation while androsterone produced only the slightest reaction. Growth of the alveoli has also been demonstrated after androgen treatment by Collip, McEuen, and Selye (25). Reece and Mixner (146) showed that, simultaneously with the stimulation of the alveoli by androgens, the prolactin content of the pituitary increased.

Studies by Leonard (109) indicated thickening of the mammary ducts by testosterone propionate in hypophysectomized male rats.

### 3. Other Endocrine Glands

In addition to the effects of the androgens on the secondary sex characteristics of males and females, it has been demonstrated that the male sex hormones have profound influences on other endocrine glands such as the pituitary, the adrenals, the thyroid, and the parathyroid.

*a. Pituitary.* The influence of androgens on the pituitary is confined to the anterior lobe. After castration, the anterior pituitary enlarges and shows cellular changes characterized by large vacuolated basophilic cells known as "castration cells" (108). The administration of androgens prevents the formation of these cells if injections are started immediately after operation. Some thirteen steroids, including  $\Delta^4$ -androstenedione-3,17 and testosterone, show this activity (3,135,136).

Men with primary testicular insufficiency usually excrete excessive amounts of gonadotrophic material which appears to be primarily follicle-stimulating hormone. Testosterone propionate administered in therapeutically effective doses (approximately 20 mg./day) reduces this excretion to values of normal men or below. After cessation of androgen treatment, gonadotrophic titers rise to the pretreatment level (20).

When female rats are treated with androgens from birth, the ovaries show an arrest of both follicle and corpus luteum formation, showing that the entire gonadotrophic production of the pituitary is arrested (104). Adequate concentrations of androgenic hormone have been shown to be capable of decreasing the gonadotrophic hormone content of the pituitary in female rats. This is due particularly to a decrease in the content of the luteinizing hormone (95). However, variable effects have been observed depending upon the concentration of androgen administered. Under certain conditions in female rats, it is possible to inhibit the production of luteinizing hormone while apparently increasing the production of follicle-stimulating hormone.

In male rats certain concentrations of testosterone propionate caused increases in the size of the pituitary but decreases in gonadotrophic hormones since the testis atrophied (188).

The pseudopregnancy reaction in rabbits, which consists of ovulation,

corpus luteum formation, and progestational changes in the uterine mucosa, normally is produced by the gonadotrophic hormone of the pituitary. This reaction can be inhibited by adequate treatment with testosterone propionate which apparently acts by suppressing the formation and release of gonadotrophic hormone (203).

b. *Adrenals.* The influence of gonadectomy on the adrenal cortex has been studied in a variety of species of animals for varying lengths of time. Certain trends are discernible but results do vary with species, age of operation, sex, and interval between operation and study of the gland. In general, the influence of gonadectomy is reflected in changes in the adrenal cortex and in mice, particularly in that portion known as the X-zone. On the basis of histological stains, a portion of the cortex appears to be different from the surrounding tissue. This zone has been observed in the adrenals of mice especially during immaturity of the animal, and tends to disappear at sexual maturity. After prepuberal castration in the male mouse, the X-zone persists. Working with concentrates of androgenic material, Martin (123) showed that androgens tend to cause the premature disappearance of this zone in immature male mice. Working with pure androgens, testosterone, androstanediol-3 ( $\alpha$ ), 17( $\alpha$ ), dehydroisoandrosterone, and  $\Delta^4$ -androstenediol-3( $\beta$ ), 17( $\alpha$ ), various workers have been able to confirm the early findings and extend them to castrated male mice, adult females, and adult ovariectomized females (31,176). In these experiments, it is difficult to say whether the influence is directly on the adrenal or through the anterior pituitary. Gonadectomy in the female mouse has no effect on the growth or involution of the X-zone.

In the rat, gonadectomy in the male is followed by hypertrophy of the adrenal cortex whereas ovariectomy in the female results either in no change or in a hypotrophy of the adrenal cortex. The X-zone of the rat is poorly developed. Howard (65) was unable to find changes in the immature rat's adrenal cortex as reported for mice.

The administration of androgens to normal male rats does not appear to influence the size of the adrenals, but similar treatment of females tends to decrease the adrenals to approximately the size in the untreated males (91,121). In castrated male rats, the administration of a variety of androgens such as androsterone, testosterone, dehydroisoandrosterone, and  $\Delta^4$ -androstenedione-3,17 prevents the usual castration hypertrophy of the adrenal cortex (50). The influence of androgens in ovariectomized female rats is much less intense but in the same direction as in castrated males.

A direct influence of the androgens on the adrenal cortex has been shown to exist in hypophysectomized rats. Leonard (110) has shown that testosterone propionate, androstanediol-3( $\alpha$ ), 17( $\alpha$ ), and  $\Delta^4$ -androstenediol-3( $\beta$ ), 17( $\alpha$ ) are capable of decreasing the rate of atrophy of the adrenal cor-

tex in hypophysectomized rats. The ability of androgen to stimulate the adrenal cortex was correlated with the activity of the androgen in stimulating the testes and prostate. The partial maintenance of the adrenal cortex in the later experiments was due mostly to increases in cell size and not to an increase in the number of cells in the cortex. In another report it was shown that, although a partial maintenance was possible for about 15 days, after that period the size of the adrenal cortex decreased in spite of continued treatment (106).

*c. Thyroid and Parathyroid.* The influence of androgens on the thyroids and parathyroids has been studied by the use of the colchicine technique. It was found that testosterone propionate caused increased cell division and apparently increased function of both these glands in immature female rats. The increase in mitotic activity was evident within the first 24 hours after androgen treatment in both the thyroid and parathyroid. It is quite likely that the changes observed are not the result of direct influence of androgens on these two glands but rather are mediated through the pituitary gland.

In adult rats, it has been claimed that chronic androgen treatment caused a decrease in weight of the thyroids in males and an increase in weight in females. In the female, the thickness and height of the thyroid cells were increased to resemble colloid adenoma (13).

#### 4. Various Organs and Tissues

*a. Kidney.* The size and function of the kidney is in part dependent upon levels of androgens in the blood. These relationships have been established by studies on the influence of castration, the administration of androgens to castrated males and normal females, and by studies on the influence of androgens on kidney function. Castration in male rats tends to produce decreased-sized kidneys. Administration of androgenic material prevents the decrease in size, or restores the kidney to normal. Similar kidney-stimulating effects have been described for castrated male mice and normal female mice. The increase in weight of kidneys as a result of androgen treatment appears to be due to marked hypertrophy of the epithelial cells of the proximal and distal convoluted tubules. The cortex is thickened and the epithelium which lines the parietal lamina of Bowman's capsule is hypertrophied (100,167,168).

Lattimer (105) found a renotrophic effect for testosterone in dogs and rats. He found a true increase in tissue solids due to an increase in cytoplasm rather than an increase in nuclear elements. Renal function in dogs as measured by inulin and diodrast clearance was increased in proportion to the increase in renal tissue. Selye has studied the physiological aspects of the kidney under the influence of testosterone. He was able to protect

mice against a large dose of mercuric chloride with pretreatment with testosterone propionate. In other series of experiments, Longley was able to protect rats against mercuric chloride when the testosterone propionate was administered after the poison (117).

Kochakian has reported strong renotrophic activity in castrated mice for testosterone, testosterone propionate, 17-methyltestosterone, androstanol-17( $\alpha$ )-one-3, androstanediol-3( $\alpha$ ), 17( $\alpha$ ), and 17-methylandrostanediol-3( $\alpha$ ), 17( $\alpha$ ). Doses of the most active compounds as small as 15  $\mu$ g./day for 30 days caused restoration of the castrated-mouse kidney to the size of that of the intact animal. Kochakian (85) also studied the influence of undernutrition on the castrated- and intact-mouse kidney. He reported that during undernutrition, the castrated-mouse kidney decreased less in size than the kidney of the normal mouse. Androgens produced less stimulation in the kidney of the underfed, castrated mouse than in that of the well-fed castrated mouse, in spite of the fact that the action of the androgens on the accessories was identical. Beland, Masson, and Selye (6) have studied a variety of androgens for their renotrophic activity.

The studies of Kochakian and Fox (88) and Kochakian (85) indicate that androgens cause a decrease in "alkaline" phosphatase/g. kidney tissue and a slight increase in "acid" phosphatase/g. tissue. No significant effect on the liver or intestine was found.

A further interesting phase of the work of Kochakian has been the study of the relationship of androgens to the concentration of arginase in the kidney. Increases in arginase concentration up to 600%/g. kidney tissue were noted in castrated mice after the administration of methyltestosterone. Testosterone, 17-methylandrostanediol-3( $\alpha$ ), 17( $\alpha$ ), and androstanol-17( $\alpha$ )-one-3 showed good response. No comparable effects in arginase activity were observed in the liver or intestine. A significant point was the fact that with testosterone there was a tendency for the concentration of arginase/g. kidney tissue to continue increasing even at a time when the size of the kidney was not further increased with larger doses of androgens.

*b. Liver.* In early studies on castrated rats, decreases of 11 to 18% in liver weight-body weight ratios were found. Histologically the liver cells from castrated animals were more vacuolated than those of normal animals (90). After castration such androgens as androsterone, androstanediol-3( $\alpha$ ), 17( $\alpha$ ), dehydroisoandrosterone, and testosterone restored the liver size to normal (51). The administration of testosterone produced changes in the liver size in normal rats. Bates, Riddle, and Lahr (4) were able to produce increases up to 60% in adult pigeons after the administration of androsterone.

*c. Heart.* The influence of androgens on the heart is not without interest, particularly since recent claims have been made for the use of testosterone

and methyltestosterone in the treatment of angina pectoris. From the work of Korenchevsky *et al.* (99), it appears that after castration in the rat there is a decrease in the size of the heart, as well as a decrease in the potential energy of the heart muscle. The administration of either androsterone or testosterone restores both the weight of the heart and the potential energy of the heart muscle.

Testosterone propionate has been reported to raise glycogen, adenyl pyrophosphate, and phosphagen content the heart muscle of the castrated rat to values in the range of normals (165).

*d. Thymus.* Castration has been shown to prevent the normal puberal involution of the thymus (44,63,122). Androgens cause premature involution of the thymus in the immature animal. It has been shown that androsterone, testosterone, and dehydroisoandrosterone are active in this respect.

*e. Hair.* The distribution of hair in humans is at least in part related to the androgens. Head hair, facial hair, and pubic hair make up the most striking group which appear to be related to the concentration of circulating androgenic substances. With respect to head hair, we have the observations that the hairline on the forehead of matured men is interrupted by two wedge-shaped indentations (*calvities frontalis adolescentium*) one over each lateral frontal region, while the hairline of women and children has the form of an uninterrupted bowlike curve. Further, in the castrated and eunochoid male the hairline conforms to the pattern seen in women; and in women suffering from adrenal tumors, with high concentrations of androgens in the body fluids, a hairline similar to the male pattern is found. In some cases of adrenal tumors of long standing, women may even exhibit advanced forms of baldness which, as has been shown, never appears in men in the absence of adequate concentrations of androgenic substances.

The association of the shape of the superior border of the pubic-hair-covered area with androgens is rather good. Thus, in women the superior border is usually horizontal while in mature men there is usually an extension of the terminal hair from the pubic area along the linea alba to the umbilicus, or beyond.

Facial hair is definitely related to androgens. The usual male beard is missing in men suffering from hypogonadism and may be actually encouraged by the administration of adequate dosage of testosterone propionate. In women with adrenocortical hyperactivity, facial hair may become so abundant as to require daily shaving. After the removal of the cause facial-hair growth may decrease in amount and coarseness.

Voice changes in humans have been correlated with the androgens; thus hypogonadal males who possess high-pitched voices, quite unlike the normal, matured man, may show signs of voice deepening and lowering of

absolute pitch with the administration of adequate concentrations of androgenic substances.

*f. Skin Pigmentation.* The relationship of skin pigmentation to androgens is another example of the diverse effects of the androgenic substances in the dynamic interplay of body processes. Hamilton and Hubert (55) have observed the inability of castrated humans to tan under the influence of sunlight. However, if such an individual were treated with testosterone propionate tanning would result. Further, tan or pigmentation in treated hypogonadal men decreased or bleached when testosterone was withdrawn and reappeared without further exposure when androgen therapy was again instituted.

#### IV. Influence of Androgens on Protein, Carbohydrate, and Electrolyte Metabolism

Anabolic effects of the androgens have been studied in some details since the demonstrations by Kochakian (82,86,87) that urinary extracts containing androgenic material,  $\Delta^4$ -androstenedione-3,17, and testosterone were capable of causing nitrogen retention in the castrated dog. Fecal nitrogen excretion remained unchanged and no increase in nitrogen was found in the blood, while the urinary nitrogen decreased. This indicated a true storage of nitrogen of about 0.05 g. nitrogen/kg./day, probably in the form of protein during the period of androgen treatment. Cessation of treatment resulted in an increase in nitrogen excretion exceeding for a short period the pretreatment levels.

At about the time of Kochakian's experiments, Papanicolaou and Falk (141) reported that the temporal muscles of male guinea pigs, castrated before puberty, remained small and similar to the muscles of the female guinea pig. The administration of testosterone propionate produced a distinct hypertrophy of the temporal muscles in the castrated male as well as in the intact and spayed female guinea pig. These experiments again indicated the ability of the androgens to cause nitrogen retention, in this case for the formation of protein for muscle.

From the experiences of Kenyon (76) and McCullagh and Rossmiller (118) it became clear that treatment with androgens such as testosterone and methyltestosterone caused weight gains in eunuchoid individuals. A progressive weight gain was seen for as long as 40 to 70 days, at which time a plateau was reached in spite of continued treatment. If the changes following androgen treatment in the eunuchoid are closely followed, it is observed that on a constant diet and regulated activity a weight gain occurs along with retention of nitrogen, inorganic phosphorus, sulfate, potassium, chloride, and sodium. Usually a decline in urine volume is seen. On cessation of treatment, the excretion of these constituents increases.

Kenyon and co-workers (79) have pointed out that the retention of sodium chloride was analogous to the action of adrenocortical steroids but retention of potassium appeared to be a distinctive phenomenon probably associated with protein metabolism.

Kochakian (83) has shown that the protein-retaining property of androgens takes place in the absence of the pituitary and testis in the dog. That the adrenal cortex is not necessary for the nitrogen-retaining effect of testosterone in humans is strongly indicated by the work of Kenyon *et al.* (79). These workers were able to demonstrate retention of nitrogen, phosphorus, sulfur, sodium, and potassium with gains in body weight after the administration of testosterone propionate to Addison's disease patients maintained on high-salt diet supplemented with daily injections of desoxycorticosterone. Kenyon *et al.* (79) studied the nitrogen-retaining effects of testosterone in normal men and women. In two normal men this group of workers observed a nitrogen retention of about half that found for the eunuchoids, while the two normal women showed irregular results. The nitrogen retention observed under the influence of the androgens represents more than mere growth of the genital tissues. The relationship between the androgens and growth somewhat clarifies the picture of the unusual, body growth associated with masculinizing tumors in children. Wilkins, Fleischmann, and Howard (197) were able to produce increase in both weight and height with methyltestosterone in dwarfed girls and boys.

A tabulation of the protein-anabolic activity of steroid compounds in man has been presented through the efforts of Reifenstein (147). This tabulation includes experiments up to December of 1942, and summarizes some 152 experiments, using fourteen steroids, by eight different groups of workers. The laboratories are those of Albright, Bassett, Kenyon, Ropes, and Bauer, of Shorr, Wilkins, and Fleischmann, and of Williams and Talbot. The types of patients studied include those with a variety of diseases, including endocrine diseases, as well as normal men and women. The compounds reported in this summary include androstanediol-3( $\alpha$ ), 17( $\alpha$ ), calciferol,  $\Delta^5$ -androstenediol-3( $\beta$ ), 17( $\alpha$ ), dehydroisoandrosterone, 17-methyltestosterone,  $\Delta^4$ -androstenedione-3, 17, diethylstilbestrol,  $\Delta^5$ -pregnenol-3( $\beta$ )-one-20, androsterone, estradiol, progesterone, anhydrohydroxyprogesterone,  $\Delta^5$ -methylandrostanediol-3( $\alpha$ ), 17( $\alpha$ ), and testosterone. From this summary it appears that methyltestosterone, testosterone propionate, and estradiol benzoate produced the greatest nitrogen retention, while estradiol dipropionate, androstanediol-3( $\alpha$ ), 17( $\alpha$ ), anhydrohydroxyprogesterone,  $\Delta^5$ -androstenediol-3( $\beta$ ), 17( $\alpha$ ), and methylandrostanediol-3( $\alpha$ ), 17( $\alpha$ ) produced nitrogen retention of a lower order. The other compounds studied appeared to be very weak or entirely inactive. From the data, it appears that the amount of nitrogen retained under the influence of a given com-

pound is dependent, within limits, upon the amount of nitrogen intake. That is, the greater the nitrogen intake the greater the retention and the shorter the period necessary to produce a maximum nitrogen retention. In young individuals, there is a tendency toward a greater nitrogen retention than in older individuals. Normal individuals show a lower nitrogen retention than that found in such conditions as hyperthyroidism, panhypopituitarism, dwarfism, Addison's disease, and eunuchoidism.

Kochakian (85a) has recently presented a comprehensive review on the protein-anabolic effects of steroid hormones. This review deals with protein-anabolic effects in dogs and rats as well as the studies in man. The early experiments on the castrated dog by Kochakian have already been mentioned. Testosterone as well as the acetate and propionate have been shown to cause nitrogen retention. Large doses of  $\alpha$ -estradiol (5 mg.) and estrone (15 mg.) were also active, but  $\Delta^5$ -androstenediol- $3(\beta),17(\alpha)$ , 1,2-cyclopentenophenanthrene, cholesterol, 7-ketocholesteryl acetate, and progesterone were inactive in the dog. A limited number of experiments have been reported in the rat (85a). Adequate doses of testosterone propionate produce nitrogen retention but about 1 week after injections are started the nitrogen excretion returns to normal in spite of continued androgen treatment.

Creatine and creatinine metabolism may be influenced by androgens. Creatinuria which follows castration in rabbits may be abolished by the administration of androgens. The excretion of creatine in normal male rabbits is decreased by the administration of testosterone propionate whether or not creatine is administered (198). Similar effects have been reported in the rat (24a), the monkey (70a), and man (64a). Methylated androgens such as methyltestosterone produce creatinuria as opposed to the creatine retention produced by testosterone (197). Testosterone propionate causes an increased excretion of creatinine after a relatively long latent period, which may be a reflection of the increased muscle mass produced by the hormone (162a).

In an early study, the claim was made that the administration of testosterone propionate to male rabbits causes hyperglycemia accompanied by a decrease in liver glycogen (36). In a later work it was shown that there was a decreased tolerance to oral and intravenous glucose after intensive methyltestosterone treatment. Liver glycogen levels were decreased. However, treatment with testosterone produced no change in tolerance. After removal of the pituitary or the thyroid in the rabbit, no effect on either glucose tolerance or liver glycogen could be demonstrated (111).

## V. Influence of Androgens on Enzymes

D-Amino acid oxidase activity of kidney shows distinct decreases after castration. In specific studies, this has been shown for oxidative deamina-

tion of D-alanine. D-Amino acid oxidase activity of this tissue is increased on administration of testosterone propionate, an increase above normal values being possible (24).

Avidin formation in the oviducts of chicks may be increased by the administration of testosterone propionate. This effect is similar to the effects produced by estrogens, progesterone, and desoxycorticosterone (64).

Castration in mice causes a decrease in kidney size which is accompanied by a proportionate decrease in both "alkaline" (pH 9.8) and "acid" phosphatase (pH 5.4). On the administration of androgens such as testosterone propionate to castrated as well as normal mice, a decrease in "alkaline" phosphatase and an increase in "acid" phosphatase in the kidney is found. In at least two tissues, the intestines and liver, no changes were observed after androgen administration to castrated animals. The decrease in alkaline phosphatase was found in total amount as well as on a weight basis. The acid phosphatase increased in total amount but remained the same or slightly increased per gram tissue (85b).

Although castration in the mouse does not affect the total arginase concentration of kidney, there is an increased amount of enzyme per gram tissue. The administration of a variety of androgens produces increases in arginase concentration. Those androgens which produce the greatest increases in arginase also cause the most profound decreases in urinary nitrogen. The greatest increases in kidney arginase were found with methyltestosterone and testosterone. Kochakian has reviewed the influence of androgens on phosphatases and arginase (85b). The serum cholinesterase of sexually mature male guinea pigs decreases after castration but may be brought back to normal by subcutaneous implantation of testosterone (195).

Torda and Wolff (180) have studied the influence of steroids on the synthesis of acetylcholine by minced frog brain. Although cholesterol did not influence the rate of synthesis, various hormones, including such androgens as testosterone, dehydroisoandrosterone, methyltestosterone, and  $\Delta^4$ -androstenedione-3,17, depressed the synthesis of acetylcholine. The cholinesterase concentration of brain tissue may be decreased *in vitro* (179). The frog rectus abdominis muscle, which normally is sensitive to acetylcholine and potassium ions, may have this sensitivity increased by testosterone propionate.

The prostate of the prepuberal monkey contains relatively small amounts of "acid" phosphatases as compared to that of the mature rhesus monkey. On the administration of testosterone propionate, the value of "acid" phosphatase increased as much as hundredfold (48).

Testosterone propionate may cause increases in muscle glycogen in the rabbit but no change in phosphagen and adenyl propposphate content (166).

## VI. Influence of Androgens on Tumors

Injections of testosterone propionate into virgin female mice of the C<sub>5</sub>H strain from the second to the twelfth month of life lowers the incidence of spontaneous mammary gland tumors and possibly increases the age at which tumors are caused to appear (75). Similar results with a small series of animals have been reported by Loeser (116). In ten mice of a strain with a high incidence of mammary cancer, subcutaneous implantation of testosterone propionate every 3 to 5 weeks was followed by death from cancer in four while nine of twelve controls died.

Extensive work of Lipschütz (113) has shown that androgens such as testosterone and dihydrotestosterone are capable of preventing estrogen-induced tumors in the castrated guinea pig.

Heiman (62) has been able to reduce the incidence of spontaneous tumors in female mice of the RIII strain from 52.2 to 19.4% by the subcutaneous administration of testosterone propionate. In the same series, the incidence of brown degeneration of the adrenals was reduced from 80.9 to 56.6% in tumor-free treated mice, but was not reduced in treated tumor-bearing mice.

Gardner *et al.* (41) produced lymphomas in 11.9% of 1799 mice in which pellets of one or more steroid or nonsteroid estrogens were implanted as compared to an incidence of 1.34% in control, nontreated animals. When testosterone propionate was given simultaneously with the estrogen, the incidence of induced tumors was reduced to approximately that of untreated controls.

## VII. Influence of Androgens on Erythrocytes and Hemoglobin

The rate of erythrocyte regeneration is markedly decreased after castration in the rat. Administration of testosterone propionate to operated animals tends to restore this rate to normal. The erythropoietic effect of androgens can also be elicited in hypophysectomized rats. Androgens tend to enhance the effect of cobalt on the rate of erythrocyte regeneration (37). Bone marrow studies also indicate that androgens tend to speed recovery in hemorrhagic anemia by virtue of the erythropoietic effect. Testosterone is capable of causing increases in the number of red blood cells in the fowl (177) and the castrated golden hamster. With respect to hemoglobin regeneration, a rather complex set of responses were obtained. That is, the male sex hormone tends to inhibit hemoglobin regeneration in the bled normal male and castrated female, while acceleration of hemoglobin regeneration was observed in bled hypophysectomized male, normal female and castrated male rats.

Administration of testosterone propionate or methyltestosterone to eunuchoid patients without pituitary disease resulted in increases in erythro-

cyte, hemoglobin, and hematocrit levels. When treatment was discontinued the values returned to the pretreatment level. Hypogonadal men with pituitary disease and normal men treated with androgens showed insignificant blood changes (119).

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## CHAPTER III

### Physiology of the Adrenal Cortex

By R. L. NOBLE

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## I. Introduction

Physiological studies on the adrenal cortex have been so extensive during the past 20 years that it is impractical to attempt to completely review the subject and still maintain a clear outline of the role played by these

glands in the numerous metabolic processes. Some of the more controversial matter, as well as much of the earliest work on the subject, has therefore been omitted in this chapter. Although the references cited are numerous, the list should not be considered complete. The concepts which have been stressed appear to be those based on the soundest experimental findings. The basic experimental evidence, which is generally omitted in reviews on the adrenals, has been quoted in many cases for readers who may be unfamiliar with such data. The more recent advances have also been included, but since many of these require further confirmation, it was believed inadvisable to speculate on the interpretations of such new findings. The contents of the chapter have been arranged according to the influence of the adrenal cortex on various organs and on different types of metabolism. The first half of the contents listed contain those effects which appear to be chiefly controlled by the so-called salt and water hormones of the cortex. The last half of the contents include actions which are affected primarily by the carbohydrate-influencing hormones, those containing oxygen at carbon atom 11.

Many articles and reviews have been published which contain detailed bibliographies. A number of these by different authors may be listed. The older work, up to 1930, is contained in a review by Britton (39) and in a book by Grollman (197). More recent reviews stressing different aspects of adrenal physiology include those by Ingle (289,294,295a), Pincus (459a), Sayers and Sayers (530b), Loeb and collaborators (363), Long (365,365a), Hartman (226), Kendall (328,330,332a), Pfiffner (457), Reichstein and Shoppee (474), Swingle and Remington (620), Swann (599), Haist (205), Parkes (442), Selye (558,559), Tepperman, Engel, and Long (627), and Goldzieher (188). Since this article deals with the physiology of the adrenal cortex, only passing reference is made to the extensive literature on the histological and pathological changes found to occur in the adrenals. For completeness, a brief outline of the morphology and control of the adrenal cortex has been synopsized from various reviews (29,289).

## II. Development and Control of the Adrenal Cortex

The adrenal cortex develops from mesoderm arising as a bud from the celomic epithelium covering the inner side of the fore part of the mesonephros. The epithelium immediately behind this area gives rise to the germinal epithelium from which the sex glands develop. The cortical tissue envelops the mass of chromaffin tissue representing the adrenal medulla and the glands come to lie on either side of the abdomen immediately above the kidneys. The cells of the cortex are arranged in three zones, which, from without inward, are named the zona glomerulosa, zona fasciculata, and zona reticularis. An older theory suggested that the cortical cells proliferate from the cortex, undergo further division in the zona

glomerulosa and migrate through the zona fasciculata and eventually disintegrate in the innermost zone (525a). More recent work, however, suggests an independence of the two outer zones of the cortex (20a,105a). Another zone, the X-zone, has been described between the zona reticularis and the medulla. The appearance of the X-zone is related to age and has certain sex relationships which indicate it may be related to androgenic function. Cytochemical and morphological studies have suggested that the zona glomerulosa may produce the adrenal hormones mainly concerned with electrolyte and water balance, whereas the zona fasciculata may control the hormones affecting carbohydrate and protein metabolism. The experimental evidence has been summarized by Greep and Deane (195a). The adrenal cortex of the female is larger in most species than that of the male, and this appears to be related to the differences in intrinsic sex hormone secretion. The adrenals are one of the most richly vascular organs in the body, receiving 6 to 7 ml. blood/g. tissue/minute. There is no known nervous control of the functional activity of the cortex. Regulation of the adrenal cortex appears to be predominantly through the adrenocorticotrophic hormone (ACTH) of the anterior pituitary gland. Removal of the hypophysis causes rapid and progressive atrophy of the adrenals and suitable adrenocorticotrophic extracts will restore the size and function of the gland. The adrenal cortex has been found to atrophy following the administration of cortical extract or adrenal steroids, but not after the use of various dose levels of different electrolytes. Such atrophy is associated with hypofunction. The atrophic adrenals of the hypophysectomized animal are functional to a certain extent, since death from adrenal insufficiency and associated electrolyte changes do not occur. Such glands on histological examination show characteristic staining alterations in the Sudanophobe area and there is some evidence that the zona glomerulosa, which is concerned with electrolyte metabolism, is not under the control of the anterior pituitary gland (105b,164a). Hypertrophy of the adrenals occurs following any form of noxious treatment or as a compensatory mechanism following the removal of one adrenal gland. Such hypertrophy does not take place in the absence of the pituitary gland. Adrenal hypertrophy may follow the ingestion of some diets high in protein (295,628). The regulation of secretion of pituitary adrenocorticotrophin (ACTH) may be related to the concentration of cortical hormone in the body fluids (530a,b) or to the secretion of adrenaline (365a). That the blood sugar level may act as a control of ACTH may also be suggested from recent findings (1a,132).

### III. Adrenocortical Insufficiency

It is about a century since Thomas Addison in 1849 presented a paper which aroused the interest of physicians in a syndrome attributed to de-

struction of the adrenal glands (1c). Studies on adrenal extirpation in experimental animals were conducted shortly afterwards by Brown-Séquard (54,55). Since that time the failure of adrenalectomized animals to survive and the signs and symptoms before death have been noted by numerous workers whose papers are reviewed by Grollman (197). For many years only survival tests were available for experiments designed to extract an active principle from the adrenal, and the potency of the first active extracts was demonstrated in this manner. The effects of cortical insufficiency on an animal are so numerous and so many systems are involved in such a complex manner that it is necessary to subdivide these actions to discuss them in detail. A brief summary has been made by Ingle (294), and has been tabulated to serve as an introduction to the more detailed consideration of the physiological changes occurring after adrenal removal.

#### EFFECTS OF ADRENAL REMOVAL

##### *Digestive*

1. Loss of appetite.
2. Delayed or incomplete absorption from intestines.
3. Nausea; vomiting. Melena; ulceration of stomach, intestines.
4. Diarrhea.

##### *Circulatory*

###### *Physical*

1. Hemoconcentration—incr. red blood cell count, volume; incr. hemoglobin.
2. Decreased blood pressure.
3. Decreased blood flow.
4. Decreased blood volume.

###### *Chemical*

1. Decreased Na, Cl, bicarbonate, glucose in serum.
2. Increased K and nonprotein nitrogen.

##### *Tissue*

###### *Physical*

1. Muscular asthenia.
2. Reduction in muscle mass.

###### *Chemical*

1. Decreased Na in muscle cells.
2. Increased K and water in muscle cells.
3. Decreased glycogen in liver and muscles after fasting.

##### *Renal*

1. Increased excretion of Na, Cl, and bicarbonate.
2. Decreased excretion of K and total N.
3. Inability to excrete ingested water.

*Growth*

1. Hypertrophy of thymus and lymphoid structures.
2. Cessation of body growth.
3. Loss of weight.
4. Fall in body temperature.

*Resistance*

1. Decr. to all forms stress, toxins, infections, trauma, environmental changes.
2. Death in untreated animals.

#### A. SURVIVAL AFTER ADRENALECTOMY

There is little doubt that removal of all functional cortical tissues is followed by death in nearly all species of animals, provided that the animal is not fed a diet high in salt or that it does not possess luteal tissue secreting progesterone. In most species death occurs within a week after the complete operation, and is preceded by gradually increasing debility of the animal. Typically an animal recovers readily from the operation, but within a day or two shows a progression of the symptoms listed. In early reports on survival after adrenalectomy confusion occurred on the importance of the adrenals due to the fact that studies were made on animals in which accessory cortical tissue was unknowingly present, or because of a lack of knowledge of the importance of the control of the inorganic constituents of the diet. On the other hand, in many of the older experiments in which death followed rapidly after adrenal removal, the severity of the operative procedure and the various detrimental environmental conditions were often underestimated as factors causing mortality. Most of the studies on adrenalectomized animals have been conducted on the rat, cat, and dog, although the rabbit, guinea pig, opossum, ferret, ground hog, pigeon, goat, monkey, duck, toad, and frog have been successfully adrenalectomized despite the difficult surgical technique. The ability of extracts to delay or prevent death of the adrenalectomized animal was one of the first methods developed for assay of cortical extracts. Many similar methods have now been reported, although some of these depend on observations of the changes occurring in some of the chemical constituents of blood or urine. In any consideration of the changes induced by adrenalectomy it should be emphasized that exposure to sudden changes in temperature, or transitory deprivation of food or water may cause a rapid deterioration in the animals' condition and an exaggeration of the biochemical changes.

##### 1. Rat

The effects of removal of the adrenal glands in the rat have probably given rise to more inconsistent results than in any other species. Many

of the controversial reports are reviewed by Britton (39) and Gaunt (169). The explanation of mortality figures which are varied from 10 to 100% now seems dependent upon variations in the completeness of adrenalectomy, the use of strains of rats with accessory adrenal tissue, the inadvertent supply of a high salt intake in the diet, and the differences in age of the animals studied. Most early reports on adrenalectomy of the rat report a high percentage of survivals, but in 1930 Pencharz, Olmsted, and Giragossintz (448,449) reported that all operated animals died. This was confirmed by Freed, Brownfield, and Evans (158), and the success of these observers in producing a high mortality was ascribed to the removal of the adrenal pedicle and surrounding fatty tissue. Firor and Grollman (152) and Schultzer (536) have confirmed this view, but Gaunt believed other factors, such as the strain of rat, were of greater importance in influencing survival (169). That certain strains of rats survive adrenalectomy better than others has now definitely been established, and this difference would seem to be related to the extent of accessory tissue present (77,169,175,183,395). In one report four different strains of rats used showed a 95% mortality within 21 days with an average life span of 7 days; with a fifth strain, however, 50% remained alive after 30 days (169). Similar changes have also been reported by other workers for different strains of rats (77,175). Richter (491,499a) has reported the interesting finding that the wild rat has adrenals three times the size of the domestic animal and is extremely sensitive to adrenalectomy and resistant to subsequent therapy. In cases in which care has been taken with the operative technique and yet mortalities of only 10% have been reported (2,397), it seems likely that the high salt or low potassium content of the diet was chiefly responsible for survival (77). The age of the rat is an important factor in survival after adrenalectomy, as was first emphasized by Kutz (343). He and others have shown that, whereas the mortality in rats 3 to 4 weeks of age weighing 40 to 60 g. is usually 100%, with death taking place in 5 to 7 days, older animals of the same strain may tolerate the operation and survive indefinitely (65,77,152,186,584,678).

Despite the larger size of the adrenals in the female rat there seems to be little sex difference in the ability to withstand adrenalectomy, provided pregnant rats are not used (197,502,584).

Some observers have noted that feeding bread to adrenalectomized rats resulted in a reduced mortality (77,81,600), but this is believed to be due to the low potassium content of the diet (728).

*Assay Method.* The use of the adrenalectomized rat as an assay for cortical extract has been frequently reported (33,65,101,169,199,339,340, 439,449,570,584). The operative technique has been described in detail (152,200,490,536), and following complete removal of the adrenals the

animals should show a progressive loss in weight as cortical insufficiency increases (246,584). Grollman and Firor (202), first suggested that a rat unit of extract should be considered as the amount which, when given as a single, intraperitoneal, daily injection to 40–50 g. adrenalectomized male rats, would allow a normal weight gain for 7 days. Death should follow withdrawal of treatment in all cases, since survival indicates the development of accessory adrenal tissue. Various modifications of this test have been made from time to time. In general it may be stated that satisfactory assays can be made only when a high percentage of the adrenalectomized control animals die within a short period on the control diet. Immature rats should be used. In such experiments the control animals should receive injections of a salt solution corresponding in concentration to that of the extract, since it has been found that 1 ml. 0.9% sodium chloride daily increased the average survival time from 5.7 to 7.7 days and double the amount of salt gave a survival of 9.3 days (537). The extract to be tested is usually injected once or twice daily for periods of 1 to 3 weeks. The efficacy of treatment is judged by the percentage of animals surviving and the increase in body weight. Units of extract are described which require 80% survival of treated animals and normal growth up to 3 weeks. An arbitrary growth of 1 g. increase daily for 20 days and 80% survival, as adopted by Cartland and Kuizenga (65), is a sensitive and commonly used criterion of activity.

A somewhat different modification of the rat survival test has been used by Waterman (671). In this the animals receive a single injection of the test extract on the second postoperative day, and the length of survival is compared with that of control, untreated animals. Assays based on the growth of the adrenalectomized rat are best suited for studies with cortical extract, desoxycorticosterone acetate (DCA), or the amorphous fraction. The results with steroids containing oxygen at carbon 11 may be unsatisfactory, since at some dose levels they may cause weight loss and yet maintain the animal in a vigorous condition.

## 2. Dog

Although removal of the adrenal glands in this species is a more difficult operation, it is readily performed and extensive physiological studies have been reported on adrenalectomized dogs. The adrenals are probably best removed in two separate operations, separated by 1–2 weeks. A one-stage bilateral adrenalectomy is followed by a high mortality rate unless immediate hormone treatment is instituted (160,445,501,502,606). Care must be taken not to injure the surrounding nerve or vascular structures, as this increases operative mortality (160,334,445). Spinal anesthesia or local anesthetic infiltration of the gland and adjoining tissues decreases the

operative risk and may make a one-stage operation practical (152,334). Following adrenalectomy, dogs which are not maintained by salt or extract show many of the typical signs of adrenal insufficiency. The animals appear normal for a few days and then stop eating, lose weight rapidly, and die (502). The average time of survival of dogs is approximately 1 week (39). One of the earliest and most consistent biochemical findings is the increase in blood nonprotein nitrogen and urea, occurring with the deterioration in the dog's condition. The prevention of this change has frequently been used as a method of assay, since it was first suggested in 1931 (217,218), and will be discussed in a subsequent section. In using adrenalectomized dogs for assay some authors feel that such factors as whether the animal maintains its appetite and body weight, shows signs of vomiting, or a fall in body temperature are of major importance and should be considered alone or with the changes in blood urea (458,661, 662,674). However, even when blood urea changes are used as a criterion for assay, it has been found that under optimal conditions the requirements of individual animals for cortin may vary 25% from time to time.

### 3. *Other Species*

Many other varieties of animals have been successfully adrenalectomized and some of the details may be found in reviews by Britton (39) and Firor and Grollman (152). Some of the more interesting findings may be noted.

a. *Cat.* The adrenalectomized cat has frequently been used in physiological studies. This species apparently stands the operation somewhat better than dogs, but death after an average of 5-10 days occurs (15,20,41, 43,88,395,720). Cats are less prone to show vomiting and peptic ulceration than dogs (506). Hartmann and collaborators have studied adrenalectomized cats extensively, and have used them for cortin assays. A cat unit has been defined as the daily dose of extract needed to maintain an adult adrenalectomized cat in good health, without any salt supplement (240). Changes in blood urea in this species, unlike dogs, are of less value as an index of insufficiency than observations on the general condition. Prolonged survival occurs only occasionally in untreated adrenalectomized cats, so that accessory tissue is an unusual finding (20,395).

b. *Guinea pig.* These animals have been advocated for studies of the adrenal, since they show a uniform response after a two-stage operation and do not have accessory tissue. The cortin requirements to maintain an animal are comparatively low. The operation described requires considerable skill because of the nearness of vascular structures (533,580). Animals having both glands removed die in an average of 4 to 5 days (69,533,580), and seldom survive 7 days. A guinea pig unit has been defined by Schacter and Beebe (533) as the amount of extract per kilogram

which, when injected in a dose of 1 ml. daily will keep an adrenalectomized guinea pig in normal condition for 10 days. In one report this unit represented 45 g. of whole fresh ox adrenals.

*c. Rabbit.* This species survives a two-stage adrenalectomy for a longer time than guinea pigs, and death occurs in 10-11 days. Accessory tissue growth, however, frequently occurs, and a high percentage of operated animals survive indefinitely (20,152,395).

*d. Monkey.* The operation in monkeys is readily performed and operated animals do not survive more than 5 to 7 days (42,152,320).

*e. Mouse.* Adrenalectomized mice show a high mortality from adrenal insufficiency and have become a standard preparation for assay of carbohydrate activity of adrenal hormones.

*f. Birds.* Pigeons, chickens, and ducks have been successfully adrenalectomized. They die rapidly in 6-15 hours unless extract or salt is given to prolong life (59,258,443). Adrenalectomized ducks have been suggested by Bülbbring (59) for use in assaying cortical extract. Birds apparently require large amounts of extract given at hourly intervals in order to withstand the operation and survive.

*g. Frog.* Adrenalectomy of the frog is usually complicated by damage to the kidney and it has not been possible until recently to prevent death by use of the cortical compounds (393,620). Clark, Brackney, and Miliner (70), however, have described a practical operative technique and were able to maintain their animals on DCA. They review the previous literature on this subject.

*h. Marmot and Opossum.* In animals which hibernate, such as the marmot, it has been shown by Britton (40,41) that adrenalectomy during the quiescent stage does not cause ill effects, but when the animal becomes active in the spring signs of adrenal insufficiency are apparent, followed by death of the animal in 2 to 10 days (40,41). The marmot and opossum have the exceptional characteristic that they do not show typical changes in electrolytes following adrenalectomy. The opossum may survive for long periods after adrenal removal, especially when a two-stage operation is performed (244,578).

#### *4. Effect of Pregnancy and Pseudopregnancy*

In female animals the functional state of the ovaries markedly affects the length of survival after adrenalectomy. Firor and Grollman (152) found that adrenalectomized pregnant rats survived an average of 15 days, or twice as long as nonpregnant controls. Similar observations have been made on adrenalectomized pregnant or pseudopregnant cats (84), dogs (458,501,506,614,615), and ferrets (177). In the original observation of Rogoff and Stewart in 1926, calling attention to this fact (501), twelve

pregnant dogs survived for 22 days after adrenal removal compared with a survival time of 7 days for nonpregnant controls. Pseudopregnancy similarly confers protection on the adrenalectomized animal. It seems clear that this effect is dependent on functioning corpora lutea in the ovary and the internal secretion of progesterone. It would be expected, therefore, that any hormone preparation producing an increased secretion by the corpora lutea of the ovary would enhance this action. This has been shown in the case of the rat by D'Amour and D'Amour (100), who produced ovaries of some 200 mg. in weight in immature rats by suitable treatment. Following adrenalectomy these animals showed a 50% survival time of 20 days against 5.2 days for controls. Similar results have been reported for mice by Pfeiffer and Hooker (456). Further experiments on this subject have been reviewed by Parkes (442). Evidence that progesterone may effectively prolong the survival of an adrenalectomized animal has been found by many workers (35,89,90,133,153,177,178,195,538,682,683). In the adrenalectomized rat doses of 0.5 to 4 mg. daily of progesterone are effective in delaying or preventing death for 15-20 days (35,133,178,195, 538). The adrenalectomized ferret can be maintained in good health by a dose of 2 mg. progesterone daily (176,177). Adrenalectomized mice are protected by 1.0 mg. daily and 50% survive after half the dose (456). Such substances as pregnanediol and allopregnanediol do not have the same action as progesterone in maintaining adrenalectomized animals (684), nor does pregnenolone (170). The action of estrogens on adrenalectomized animals is deleterious and shortens the survival time, whereas androgens such as testosterone, androstenedione, androstanediol, and dehydroandrosterone are less toxic but are not beneficial (176,177, 443,456,591).

#### B. METHODS OF ADMINISTRATION OF CORTICAL HORMONES

In the treatment of animals and humans with adrenal hormones various forms of administration have been used. These general procedures have been briefly summarized and comments added as to their effectiveness or disadvantages. Only a few of the early references are cited. Cortical extract has usually been administered by subcutaneous, intramuscular, or intravenous injection, and, in general, the effectiveness is increased by increasing the number and frequency of injections. For certain experiments on carbohydrate storage it has been advantageous to give injections every hour. In the rat, cortical extract apparently is almost as effective for maintaining growth and carbohydrate stores when given orally, especially when placed in the drinking water (33,101,145,201,298,340,590). In the dog, however, orally administered cortin was less than 8% as effective in maintaining blood urea as when injected (458). Different opinions

have been expressed concerning the oral activity of DCA. Grollman (199,201) found that when mixed with the food for rats DCA was just as effective as when injected. Administration in oil by stomach tube was of less value. Kuizenga, Nelson, and Cartland (340) and others (159b) found oral DCA some 35 times less active for rats than when injected. In cases of Addison's disease an oral dose of DCA ten times the injected maintenance dose was not effective and was followed by adrenal insufficiency (7). Corticosterone and dehydrocorticosterone have been found as effective on oral administration as on injection (340). The use of adrenal hormones in compressed pellets implanted subcutaneously or intramuscularly, by the technique originally described by Deanesly and Parkes (106), has been found to be very effective and economical. In rats pellets made from cortical extract, corticosterone, and Compound E were found by Ingle and Mason (304) to act effectively for 4 weeks or longer. The last substance, however, lasted a shorter time. DCA pellets have been used effectively in rats (58), dogs (639), and in humans (382,641,648), and the technique has been described (639,648). In the human, pellets of 100 to 150 mg. may be substituted for an injected daily maintenance dose of 0.4 to 0.5 mg., and are effective for 9-12 months. The average daily absorption from such pellets was 0.29 mg. (641). Detailed observations on the rate of absorption in humans from DCA pellets have been reported (379,382). Anderson, Haymaker, and Henderson (7) introduced another form of DCA therapy for humans with a number of advantages over pellet administration. In this report 10 mg. per ml. was dissolved in propylene glycol, and was administered by drops placed under the tongue. This sublingual form of therapy was found equal to that by subcutaneous or intramuscular injection in six cases of Addison's disease. Other workers have confirmed the effectiveness of this form of therapy, but believe that three to four times the intramuscular dose is required for comparative effectiveness (76,83,645). The percutaneous (582,645) and rectal (645) routes of administration have also been used and comparative studies made experimentally (645).

### C. EFFECT OF TREATMENT ON SURVIVAL

#### 1. Adrenal Extracts

Until pure adrenal steroids were made available all maintenance experiments were performed with cortical extracts, and it is of interest to note that Pfiffner, Swingle, and Vars by 1934 had reported that two adrenalectomized dogs had been maintained on cortin for a period longer than 2 years (458). Since over a period of years these extracts have increased in purity and potency, it is difficult to make comparisons of the potency of different extracts. The only basis of comparison which might be con-

sidered is the equivalent amount of fresh adrenal glands used as a starting material. In many cases, however, this information is not available. Cortin preparations available today are standardized in various ways, but the more active ones have an activity of about 30 to 40 dog units (normal blood urea maintenance) per ml. 1 ml. of extract may represent about 50-75 g. tissue. The extracts are nontoxic and free from pressor or depressor substances, and so may be administered intravenously or intramuscularly. Even though two extracts may on assay have the same potency in one type of test this is no assurance that the proportion of the various adrenal steroids is the same in the two instances. It is probable that even extracts prepared in the same fashion from the same type of starting material must vary from time to time in their steroid content. The species from which the adrenal glands are obtained is important in making extracts. Hog adrenal extracts are more potent than those made from sheep or ox and contain a higher concentration of 11-oxygenated compounds, with a resulting difference in activity on metabolic processes (341a). The assay of a number of samples of cortin by four different methods and the results, expressed in terms of adrenal steroids as reference material, have been reported in detail by Olson, Jacobs, Richert, Thayer, Kopp, and Wade (439). Some of the equivalent values taken from this recent paper and compared with others obtained by different workers may be mentioned. Using the growth test in immature adrenalectomized rats, it has been found that to maintain a rate of growth of 1 g./day required a daily amount of cortin equivalent to from 40 to 75 g. original fresh adrenal tissue. This effect was equal to that found with the daily injection of 0.067 mg. DCA. A dose of 0.1 mg. of the latter substance, however, was required to support normal growth. Cartland and Kuizenga (65) found cortical extract by the same assay to contain 5-8 rat units/ml. and 1 ml. cortin represented 40 g. adrenals. In a later paper approximately the same values were found (341). Cortical extracts have also been compared in terms of DCA in their ability to maintain normal blood urea in adrenalectomized dogs (439). It was found that, with the animals on a diet low in sodium, approximately 1.0 mg. DCA was the daily requirement. When cortical extract was compared, the equivalent of approximately 0.25 g. fresh tissue was required daily. In one of the earliest reports  $\frac{1}{2}$  to  $\frac{1}{6}$  ml. cortin/g. body weight was found necessary to keep adrenalectomized dogs without symptoms for a 5 day period (217). However, in other experiments, in which 1-1.5 mg. DCA daily was required to maintain an adrenalectomized dog on a low-sodium diet, the equivalent total dose of cortin when given twice daily was 6 to 8 ml., representing 240 to 320 g. fresh cortex (635). Life was maintained by 2-5 ml. cortin daily in another report (661). In another case using two different extracts maintenance re-

quired a dose of approximately 0.5 or 1 ml. when the diet contained 2% sodium chloride (79,458). On a 1% sodium chloride diet the amount required might be expected to be approximately five times as great. Orally administered cortical extract is only about 8% as effective as subcutaneously injected extract (458), although the amorphous fraction from hog adrenals has been found to be as active when given orally as when injected (341a).

These results indicate that in terms of DCA comparatively much less extract is effective in maintaining normal blood values in dogs than for life maintenance in the rat experiments. The cortin requirements of the rat are relatively great, and in a comparison by Cartland and Kuizenga (65) 1 rat unit equaled 22 dog units. On the basis of body weight the young growing rat after adrenalectomy requires more than 300 times as much hormone as does the adult adrenalectomized dog. The same approximate difference has been suggested by D'Amour and Funk (101), who stated that a 50-g. rat requires as much cortin as a 15-kg. dog. The guinea pig requirement is apparently about 5 to 10% that of the rat in terms of body weight, or approximately the same as that of the dog (533). Cleghorn, McHenry, McVicar, and Overend (80) found that to maintain adrenalectomized cats and dogs in comparably good condition required 0.39 to 0.65 ml./kg./day and 0.036 to 0.090 ml./kg./day, respectively. 1 ml. cortical extract was equivalent to 40 g. beef adrenal. The cat therefore requires about ten times the dog dose.

## 2. Adrenal Steroids

It has been shown by many workers that of the adrenal compounds DCA is highly effective in maintaining the adrenalectomized animal in a normal condition. Since this substance is so active and readily available, it is used as a reference material for comparative assay. The amorphous fraction is probably even more active on a weight basis, but fewer tests have been conducted due to the scarcity of such material. The other adrenal steroids are definitely less effective. Using maintenance and growth of young adrenalectomized rats, DCA, shortly after its identification, was shown to be active (593), and it is an effective therapy in adrenalectomized rats even when the diet is practically devoid of sodium chloride (144). Doses of 0.5, 0.25, and 0.1 mg. show definite action (199,439).

Kuizenga, Nelson, and Cartland (340) using their rat unit have found that DCA, corticosterone, and dehydrocorticosterone contained 35, 6, and 4 units/mg., respectively. On oral administration the last two compounds were equally active but DCA had less than 1 unit/mg., contrary to Grollman's report (199) that DCA was fully effective orally. The lack of oral activity of DCA has been noted by other workers (159b). Kuizenga and

Cartland (339) also found Compound E to assay at 1-2 rat units per mg., but later results indicated a potency of eight times this figure (341). Certain amorphous fractions, however, contained 20-30 units/mg. A diet low in carbohydrate has been stated to reduce the effectiveness of DCA (145). Waterman (671), using his single-injection assay method, found doses of 1.0 to 3.0 mg. DCA active. Corticosterone butyrate was active at 1.0 mg. but the benzoate was not effective. 17-Hydroxycorticosterone was active in doses of 1.5 or 3.0 mg. but the amorphous fraction was not active in a 2-mg. dose. Because of the method used the activity is no doubt markedly influenced by such factors as the rate of absorption, and the results are not comparable to the more standard procedure mentioned above. The recently reported Lowenstein-Zwemer compound has been stated to be as active, or more so, than DCA for life maintenance (374, 374a), but there has not yet been confirmation of this work.

Selye (555) found 2 mg. daily of  $\Delta^6$ -pregnene-3,21-diol-20-one acetate (acetoxy pregnenolone) maintained the life of adrenalectomized rats, and  $\Delta^6$ -pregnen-3-ol-20-one also showed activity, although less marked. Segaloff and Nelson (550) found the latter substance inactive, but the former was markedly potent, more so than progesterone, but not so active as DCA. Pregnenin-17-ol-3-one in doses of 5 or 10 mg. given orally was ineffective. Acetoxy pregnenolone has also been reported to have about one-sixth the activity of DCA when tested on the adrenalectomized guinea pig (55b) and adrenalectomized dog (73a).

The DCA requirement of the adrenalectomized cat or guinea pig would appear to be of the order of 0.6 to 1.0 mg. daily (69, 234). Adrenalectomized mice require 0.25 mg. DCA daily to maintain normal weight when drinking water. If physiological saline instead of water is given, DCA gives rise to toxic reactions, causing death (456). The use of assays employing growth have been criticized from time to time, since in some cases animals growing well succumb to adrenal insufficiency. When individual adrenal steroids are used for assay a more serious objection may be present, as has been stressed by Kendall (332). Corticosterone and Compound E in large doses may cause a loss of weight *per se* in normal or adrenalectomized animals, although conferring protection in other respects (285, 286, 681). Growth curves obtained with such experiments could therefore be misleading. Kuizenga, Nelson, and Ingle (341), however, investigated the influence of this effect on the assay method of Cartland and Kuizenga, and believed that various doses of Compound E gave results comparable to those obtained with cortical extract. Results of assays using adrenalectomized dogs are given in the following section, since in nearly all cases blood changes in urea or nonprotein nitrogen, and not survival, are used as an indication of the effectiveness of therapy.

### *3. Adrenal Hormones and Addison's Disease*

Since the treatment of Addison's disease is a clinical problem, no attempt has been made here to review the extensive literature. Certain pertinent observations have been referred to, however, in various sections of this chapter. From the point of view of life maintenance alone, human cases of adrenal insufficiency are frequently complicated by other disease and results cannot, therefore, be compared to those on experimental animals. Before the time when the effectiveness of salt therapy became known no specific therapy existed. The demonstration by Loeb (357,358) that a high salt intake was beneficial to cases of Addison's disease has been widely confirmed, and this treatment is a standard practice in present-day therapy. The use of cortical extract in treatment has been extensive, although limited by a number of factors. Its special value at present appears to be in cases of adrenal crisis and those in which a low blood sugar is pronounced. Its intravenous action and nontoxicity enable it to be used in all acute cases. It has been used, however, in chronic cases over prolonged periods (514).

A cortical extract prepared from hog rather than beef adrenals appears to be particularly rich in carbohydrate activity (154a,377). The use of DCA in the treatment of Addison's disease has been widespread, and various methods of administration, as previously noted, have been used. Some of the numerous reports on its effectiveness and the potential dangers associated with such therapy may be found in the list of references (78,148,350, 351,380,381,524,586,631,632,634,647,648,651,655). Some of the other adrenal hormones have been used occasionally on humans (632,651) and a detailed discussion on treatment has recently been published (695).

## **IV. Biochemical Changes in Adrenal Insufficiency**

In subsequent sections of this review reference will be made to chemical changes occurring in the blood and urine after adrenal removal. In order to avoid repetition, a number of papers on this subject have been summarized and are tabulated below. In a subsequent section of this chapter on intracellular fluid and electrolytes additional information on the biochemical changes in muscle will be given. Many authors have discussed biochemical changes, and the papers selected contain results from different laboratories made over a period of years. The most complete data was presented in 1933 by Harrop and co-workers in a series of papers (219, 220,222,223). In most cases an extensive number of determinations have been made on normal and adrenalectomized animals and the effect of replacement therapy has been included. Findings in dogs, cats, rats, monkeys, and man are recorded (cf. the review of Kendall, 332a).

## A. Dogs

These observations were made by three separate groups of workers: Harrop, Soffer, Ellsworth, and Trescher in 1933 (219), Marenzi in 1938 (394), and Muntwyler, Mellors, and Mautz in 1940 (420). The changes in the blood obtained from the jugular vein of seven dogs before removal

TABLE I

Blood mineral	Partial adrenalect.	Total adrenalect.
	mg. %	mg. %
Chloride . . .	396.3	344.7 (-13.0%)
Sodium . . .	385.1	338.0 (-12.2%)
Potassium	20.3	28.9 (+42.3%)
Calcium	12.2	11.7 (-4.1%)
Phosphorus .	4.3	5.65 (+31.3%)

TABLE II

Measurement	Serum		Erythrocytes	
	Normal	Deficient	Normal	Deficient
Water <sup>a</sup> . . .	917.6	899.0	652.8	664.4
pH . . .	7.42	7.21		
Chlorides <sup>b</sup> . . .	117.2	107.4	80.5	79.6
Bicarbonate <sup>b</sup> . . .	23.1	12.5	18.0	10.8
Determined acid <sup>b</sup>	156.6	139.6	162.2	134.1
Sodium <sup>b</sup> . . .	155.9	139.6	145	128.7
Potassium <sup>b</sup> . . .	3.5	9.1	8.9	9.0
Determined base <sup>b</sup>	159.4	149.0	153.8	137.7
Protein <sup>c</sup> . . .	6.3	8.1		
Cell volume <sup>d</sup> . . .	..	..	49.4	58.3

<sup>a</sup> g./kg.<sup>b</sup> m.eq./kg. water<sup>c</sup> g./100 ml.<sup>d</sup> ml./100 ml.

of the second adrenal in a two-stage operation were compared to those in the same animals 36 to 48 hours after complete adrenalectomy (394), and are shown in Table I.

Femoral artery blood from 24 normal dogs and 11 dogs in marked adrenal insufficiency were compared as shown in Table II; chemical changes occurring in the red blood cells are also included (420).

Harrop and associates were the first to make extensive observations on

changes occurring in adrenalectomized dogs, and some of the published data (219,223) have been summarized (Tables III-VII). Table V shows the effect of withdrawal of cortical extract followed by adequate replacement therapy; the balance experiment was carried out on a dog fed meat

TABLE III

## COMPARISON OF BLOOD VALUES OF FOUR DOGS BEFORE AND AFTER ADRENALECTOMY

Plasma mineral m.eq./l	Control	Insufficiency
Calcium, . . . . .	6.1- 7.2	5.8- 8.2
Magnesium . . . . .	1.3- 1.5	1.7- 4.1
Potassium . . . . .	4.7- 6.2	9.8- 13.9
Total base . . . . .	150.6-154.4	139.0-150.4
Sodium . . . . .	137.4-140.5	112.8-132.7
Chlorides . . . . .	116.8	103.0
Bicarbonate . . . . .	23.6	14.2
<sup>a</sup>		Good condition
		Severe insufficiency
Blood NPN . . . . .	36	140
Blood lactic acid . . . . .	6.0	8.7
Plasma inorganic PO <sub>4</sub> . . . . .	4.6	10.1 (terminal rise)

<sup>a</sup> mg./100 ml.TABLE IV  
URINARY CHANGES ON FOURTH DAY OF ADRENAL INSUFFICIENCY

Measurement	Normal	Insufficiency <sup>c</sup>
Urine value, 24 hours		
N <sup>a</sup> . . . . .	5.8	3.5
Urea <sup>b</sup> . . . . .	4.8	2.9
Volume <sup>b</sup> . . . . .	123	160 (terminal decrease)
Creatine N <sup>a</sup> . . . . .	0.36	1.34
Creatinine N <sup>a</sup> . . . . .	0.28	0.23
Blood NPN <sup>a</sup> . . . . .	38	100

<sup>a</sup> mg./100 ml.<sup>b</sup> ml.<sup>c</sup> Death by 4½ days.

plus 2 to 3 g. sodium chloride daily. Tables VI and VII show the findings recorded for the blood and circulation of such dogs.

In severe insufficiency the body temperature may fall 2 to 4° F. (rectal). The respiratory volume usually increases, although the rate is unchanged. Oxygen consumption may be 20 to 25% below control levels and the respiratory quotient falls terminally.

TABLE V

Measurement	Control, adequate extract	No extract for 4 days; maxi- mum change	After daily extract injection	
			2nd day	5th day
Body weight, kg. . . . .	9.3	8.6	8.3	8.9
Food intake, g. . . . .	450	200	450	450
Daily urine, ml. . . . .	422	685	573	425
Daily fluid intake, ml. . . . .	683	668	958	703
Daily fluid balance, ml. . . . .	+261	-52 (av.)	+385	+279
Daily Na balance, m.eq. . . .	+3.8	-25.7 (av.)	+23.6	+8.2
Daily Cl balance, m.eq. . . .	+0.1	-20.9 (av.)	+23.4	+19.4
Plasma total base, m.eq./l.	156.6	..	144.6	150.6
Plasma Na m.eq./l.	142.1	134.9	134.2	137.7
Plasma Cl m.eq./l. . . .	112.2	106.0	105.2	111.7
Plasma HCO <sub>3</sub> m.eq./l. . . .	22.9	18	21.0	23.3
Blood NPN, mg. % . . . .	48	56	40	..

TABLE VI

Measurement	Control	Insufficiency	
		3 days	6 days (severe)
Pulse. . . . .	68	76	118
Circulatory minute vol., l. . . . .	0.83	0.64	0.38
Blood O <sub>2</sub> capacity, vol. % . . . .	20.4	22.7	25.5
Blood O <sub>2</sub> arterial, vol. % . . . .	19.8	22.4	25.0
Blood O <sub>2</sub> venous, vol. % . . . .	14.8	14.3	14.1
Blood arterial-venous diff., vol. % . .	5.0	8.1	10.9
Blood NPN, mg. % . . . .	..	..	132.0

TABLE VII

Femoral artery blood	Control	Insufficiency	
		4 days	7 days (severe)
O <sub>2</sub> capacity, vol. % . . . .	10.5	14.4	16.5
Hematocrit, % . . . . .	26.5	36.5	41.0
Plasma volume, % . . . . .	72.0	61.5	57.5
Red blood cells, millions . . . . .	4.24	5.07	6.09
NPN, mg. % . . . . .	34	55	184
Body weight, kg. . . . .	7.2	7.1	6.6
Plasma protein, g./100 ml. . . . .	5.26	..	6.46

## B. CATS

Biochemical findings in adrenalectomized cats have been taken from papers by Baumann and Kurland (20), Hegnauer and Robinson (252), and Darrow, Harrison, and Tafel (103). (A summary of such findings up to 1934 has been published by Zwemer and Sullivan, 727.) Normal values

TABLE VIII

Plasma	Normal	Adrenal insufficiency
Plasma, % . . . . .	64.6	57.2
Water, % . . . . .	91.3	90.0
Na, mg. % . . . . .	375	320
K, mg. % . . . . .	19	28
Ca, mg. % . . . . .	10.2	11.5
Mg, mg. % . . . . .	2.4	3.2
Cl, mg. % . . . . .	415	378
Inorganic P. mg. % . . . . .	5.8	6.3

TABLE IX

Measurement	Plasma or Serum			Erythrocytes		Ref.
	Normal	Insuf-ficient	Diff. %	Normal	Insuf-ficient	
Na, m.eq./l. H <sub>2</sub> O . . . . .	177	138	-22	162	142	252
Na, m.eq./l. H <sub>2</sub> O . . . . .	162	146	-10	...	..	103
K, m.eq./l. H <sub>2</sub> O . . . . .	3.9	5.9	+50	5.0	7.9	252
K, m.eq./l. serum . . . . .	4.3	6.6	+53	..	..	103
Cl, m.eq./l. H <sub>2</sub> O . . . . .	141	121	-16	..	..	252
Cl, m.eq./l. H <sub>2</sub> O . . . . .	129	116	-10	..	..	103
H <sub>2</sub> O, % . . . . .	91.1	91.0	..	64.7	66.5	252
H <sub>2</sub> O, % . . . . .	93.3	92.7	..	..	..	103
Hematocrit, % cells . . . . .	..	..	..	37.6	41.4	252

obtained are compared to values from animals showing moderate or severe cortical insufficiency. In the first case (20), twelve cats survived 2 to 22 days after adrenalectomy and were bled at different stages of insufficiency with the results (average) shown in Table VIII.

The results of the two other reports have been summarized in one table, since they are comparable, except that one was made on plasma and red blood cells of eight cats (252), and the other on serum of ten animals (103)

in moderately severe insufficiency. The average results are shown in Table IX.

### C. RATS

The blood findings for rats in severe adrenal insufficiency have been taken from Harrison and Darrow (212) and are given in Table X. Buell and Turner (58) studied the effects of adrenalectomy and treatment by salt or implantation of DCA pellets; their average results are shown in Table XI.

Conway and Hingerty (86a) have recently made extensive observations of changes found in muscle and blood of the adrenalectomized rat. The

TABLE X

Serum	Control	Insufficient
Water, g./100 ml.	93.9	93.8
Cl, m.eq./l.	105	97
Na, m.eq./l.	144	136
K, m.eq./l.	5.2	7.4
NPN, mg./100 ml.	35	96

TABLE XI

Serum	Intact controls; no treatment; 11 rats	Adrenalect.; no treatment; 11 rats	Adrenalect.		Intact
			NaCl, 8 rats	DCA, 11 rats	
Cl, m.eq./l.	98.4	94.0	107.3	93.4	88.4
Na, m.eq./l.	144.2	133.7	143.4	141.4	142.7
K, m.eq./l.	6.6	8.5	7.0	7.8	6.2
Ca, m.eq./l.	5.5	5.8	5.2	4.3	5.3
NPN, mg./100 ml.	34	46	48	36	34

substances determined in muscle were potassium, sodium, magnesium, chloride, phosphate, acid soluble phosphate, phosphocreatine, adenosine triphosphate, total hexose monophosphate, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate, phosphoglyceric acid, phosphopyruvic acid, carnosine, anserine, and water content. They have found that 3 to 6 days after adrenalectomy there was an increase in skeletal muscle of potassium, magnesium, phosphocreatine, and glucose-1-phosphate and a decrease in sodium, glucose-6-phosphate and fructose-1:6-diphosphate. In the same animals there was an increase in plasma, potassium, magnesium, urea, total phosphate, inorganic phosphate, and a decrease in sodium, chloride, bicarbonate, and calcium, and probably in plasma pH.

## D. MONKEY

Britton, Silvette, and Kline (42) studied blood changes in different species of monkeys. The results of seven normal and five adrenalectomized (terminal stages) white-faced monkeys are averaged:

Serum	Normal	Severe insufficiency
Na, m.eq./l.....	143.3	126
Cl, m.eq./l.....	114.7	104.6
Urea, mg.%.....	95.1	141.6
Glucose, mg. % .....	106	22.8

## E. HUMAN

Thorn, Howard, Emerson, and Firor (648) have published extensive observations on a carefully controlled case of Addison's disease during

TABLE XII

Treatment 12 g. NaCl	Period 1, cortical extract	Period 2, DCA in- jection	Period 3, no hormone	Period 4, DCA pellets
Body wt., kg.....	64.7	66.3	65.5	68.3
Blood pressure, mm. Hg	108/70	114/78	112/76	130/80
Hematocrit, % cells	44.2	41.7	45	39.7
Plasma vol., ml.	2870	3090	2760	3380
Total blood vol., ml.	5140	5300	5020	5600
Plasma Na, m.eq./l.....	143.2	139	135.5	138.1
Plasma Cl, m.eq./l.....	100.8	102.8	97.8	105.6
Plasma K, m.eq./l.....	4.0	5.8	5.4	4.0
CO <sub>2</sub> comb. power, vol. %	52.2	57	58.7	63.6
NPN, mg. %	32	28	30	28
Glucose, mg. %	80	73	84	85
Na balance, m.eq./24 hr.	+9	+103.4	-19.9	+77
Cl balance, m.eq./24 hr.	+2.4	+62.8	-19.9	+41.4
Urinary K, m.eq./24 hr.	65.5	71.5	55	63.2
Urinary P, m.eq./24 hr.	0.88	0.73	0.76	0.73

different forms of therapy given consecutively. The patient at the start was maintained in fair condition by 12 g. salt daily orally, and 2 ml. cortical extract injected subcutaneously three times daily; the biochemical findings were averaged over a 5 day period (period 1, Table XII). For a similar second period, 5 mg. DCA in oil was given as a single daily injection in-

stead of cortical extract. For a third period no hormone was given and salt was continued. The final period shows the changes following implantation of DCA pellets and a continuation of salt therapy.

Jimenez-Diaz (318) has stressed the acidosis and failure of the kidney to produce ammonia which occur in Addison's disease.

## V. Adrenal Cortex and Kidney Function

One of the most important roles of the adrenal cortex is the maintenance of kidney function and regulation of output of certain substances in the urine. The nonprotein nitrogen fractions, sodium chloride, potassium, and water excretion are markedly affected by alterations in adrenal secretion and are discussed in this section.

### A. NONPROTEIN NITROGEN AND UREA

Changes in blood nonprotein nitrogen (NPN) have been noted since serious attempts were made to study animals surviving short periods after adrenalectomy (19,30,39,239,399,602,603,620,714,722). The blood NPN and urea have been found to start to increase shortly after treatment of an adrenalectomized animal is stopped and to increase further as insufficiency becomes more acute. The severity of other symptoms and general condition of the animal more or less parallels the azotemia, but the latter is probably a more accurate end point, and may be apparent earlier than other signs (458,502). The increased blood urea and fall in blood pressure occur simultaneously and in inverse proportions (619). At the same time as the NPN and urea are increased, appreciably higher values are obtained for blood creatinine, uric acid, sulfate, and phosphate (221, 222, 239, 501, 514, 603, 623, 702). Other evidence of renal failure has been obtained by excretion tests. In 1916 Marshall and Davis (399) showed that in the cat with adrenal insufficiency there was a decreased excretion of creatinine and phenolsulfonaphthalein in the urine. Urea clearance dye excretion tests also indicate impaired renal function, especially terminally and in acute insufficiency (30,184,211,592). In dogs, however, which show only mild symptoms of adrenal insufficiency and have essentially normal blood electrolytes there is a marked fall in diodrast clearance and the maximum tubular excretion of diodrast. Inulin clearance is also somewhat below normal (689c).

A considerable number of reports have been made on kidney function in patients with Addison's disease since Smith in 1897 (585a) described a case with fatal anuria. These have been reviewed by Talbott, Pecora, Melville, and Consolazio (625). These authors report an extensive study on ten patients with Addison's disease. These cases were all considered to have chronic adrenal insufficiency, and were not studied while in acute

insufficiency. They showed a significant depression of the rate of glomerular filtration as measured by inulin clearance. Treatment with DCA caused a partial restoration of glomerular filtration but even after a year's treatment this was not normal. Cortical extract did not cause greater improvement than DCA. The clearance of creatinine was also reduced. Measurements of diodrast clearance at low plasma levels indicated a reduced renal plasma flow, which was not corrected by cortical-extract therapy. The reabsorptive capacity of the tubules for glucose was reduced. Sodium clearance was normal, whereas an increased clearance of potassium was observed following DCA treatment. Waterhouse and Keutmann (670b) have recently reported similar observations.

The degree of uremia usually found terminally after removal of the adrenals is probably not sufficient to account for the death of the animal. Higher levels of blood urea are not necessarily lethal for an animal with intact adrenal glands (104,378), although it must be remembered that the adrenalectomized animal has a much lower tolerance to toxic substances generally and also to urea (579). It might be expected that pathological lesions would be demonstrable in the kidneys of such animals. The only consistent finding would seem to be an acute lipoid nephrosis of the kidney tubules (19,19a,204,204a,239,385,501), but these changes are hardly severe or extensive enough to be wholly responsible for the impaired kidney function (184,399,602,625).

The changes occurring in the cardiovascular system of the animal in acute adrenal insufficiency would be expected to influence renal function. The low blood pressure, hemoconcentration, reduced blood flow, and low metabolic rate would tend to affect the kidney adversely. Similarly, the sodium depletion and reduction in plasma volume tend to decrease the rate of glomerular filtration. This in turn causes increased urea reabsorption, with the resulting high urea levels in the blood (211). It has been shown, however, that a defect in glomerular filtration exists even when the blood pressure is well maintained in cases of Addison's disease (625). Also the blood NPN of the adrenalectomized dog may remain high despite an increased urine volume output associated with a reduced total nitrogen of the urine (219,362). The suggestion that a diminution in efferent arteriolar tone in the kidney causes the reduced urea clearance would seem in harmony with other vascular changes associated with adrenal insufficiency (625).

As would be expected, treatment with high-salt, low-potassium intake (4) or cortical extracts which maintain the animal in normal health effectively reduce the elevated blood urea and cause an increased urea excretion. DCA has been studied with great interest in this connection because of its marked activity (234,363,524,635). The blood urea of the normal, intact animal apparently can be lowered by large doses of this substance (338,

484), and the severity of uremia in nephrectomized animals may be reduced (116,498,554,567). It is possible that these actions are explicable by the potassium depletion and depression of protein catabolism in the body caused by DCA (483,484). The blood urea of the adrenalectomized nephrectomized rat does not increase before death and its survival time is increased provided the animal has previously been fed a low-potassium diet for some days (34a).

That changes in the metabolism of the kidney cells take place after adrenalectomy has been indicated from direct experiments measuring oxygen consumption. It seems clear from a number of reports that the oxygen uptake of kidney slices is about 25% less if the animal is adrenalectomized and that cortical-extract treatment will restore this defect (96, 522, 652). Chambers and Cameron (66), found that the secretory activity of the renal proximal tubules of the chick in tissue culture could be stimulated by cortical extract or Compound E, but not by 11-dehydrocorticosterone, the amorphous fraction, or ascorbic acid.

### 1. Assay Method

The use of the blood level of NPN or urea as an index of adrenal insufficiency is one of the most accurate and frequently employed methods of assay. As originally outlined by Harrop, Pfiffner, Weinstein, and Swingle (218), a dog unit was defined as the "minimum daily kilogram dose of cortical hormone necessary to maintain normal physiological conditions in the adrenalectomized dog for a period of 7-10 days: the two criteria of normal physiological condition being maintenance of body weight and blood level of NPN or urea." In the conduct of the test the animal was given injections twice daily of an amount of extract expected to be more than adequate. The dose was reduced after each period of 7-10 days until one was reached which allowed the NPN to rise some 15 mg. to 50 mg./100 ml. In a later paper from the same laboratory (458) it was considered preferable to take the end point as a rise in blood urea of 100% irrespective of weight loss of the animal. Animals used for assay must be free of infection, intestinal parasites, and kept in a constant environment and on a constant dietary regimen if consistent results are to be expected. Dogs may be used for repeated tests over prolonged periods and give constant results. Although the cortical requirements of individual dogs may vary as much as 100%, the individual requirement varies about 25% in repeated tests. Minor changes in this test have been introduced from time to time, and usually concern the number of injections or the length of time observations are made. An accurate estimate of the amount of sodium chloride in the diet is also of importance for evaluating results, and it would seem logical to use DCA as a standard of comparison.

The control of blood NPN or urea and maintenance of the general

condition of the animal can be accomplished by diet alone or by adrenal extracts and steroids. Of the adrenal compounds the amorphous fraction is probably most active and DCA slightly less so. It has been reported that daily doses of 0.365 and 1.56 mg. DCA were required to maintain blood urea in adrenalectomized dogs of 8-10 kg. In the first case the diet contained 2% sodium chloride, and in the second 1%. This alteration in salt intake therefore made a difference of five and a half times in the DCA requirements (79,219). Other workers have found values of approximately the same order (401,439,484). Some comparative requirements of the adrenal steroids, using this method of assay, have been tabulated from different reports (159a,329,401,459), and are as follows:

Daily requirement/kg. body weight	Unit, mg.
Amorphous fraction ..	0.001-0.0025
DCA ..	0.015
DC ..	0.030-0.045
Corticosterone ..	0.100
11-Dehydrocorticosterone ..	0.125
17-Hydroxycorticosterone ..	0.35-0.500
Compound E ..	0.35-0.500

### B. SODIUM AND CHLORIDE

The importance of the adrenal cortex in the control of body electrolytes developed from observations on the beneficial effects of salt solutions in adrenal insufficiency. In older publications it was noted that injection of saline and glucose solutions sometimes improved the condition of the adrenalectomized animal, but the effects were not definitely established (39). Baumann and Kurland in 1927 (20) noted that following adrenalectomy in cats there was a lowering of plasma sodium and chloride and a rise in potassium, and it was believed that these changes intimately concerned an important function of the adrenal. Marine and Baumann (395) showed that such substances as normal saline, Ringer solution, and isotonic sodium acetate, administered to a large number of adrenalectomized cats, prolonged their survival period from 5.3 days to 13-15 days. Glucose and glycerol solutions were practically ineffective. These findings were confirmed by Corey (88). More marked effects of Ringer solution in prolonging life after adrenalectomy in dogs were reported by Rogoff and Stewart (504). They noted that even the acute symptoms of severe insufficiency rapidly responded to the injection of salts. The full realization of the significance of these observations was not appreciated until some years later when Loeb in 1932 (357,358) reported his dramatic observations in

Addison's disease. He showed that the crisis in this disease was characterized by a low plasma sodium, and that the administration of sodium chloride would keep such a case in good health (358). Following the stimulus of these studies a great many papers have been published on carefully planned balance experiments in which alterations in sodium, chloride, potassium, NPN, water, urine constituents, etc. have been estimated (364,516,579,630,636,642,644).

Extensive studies on dogs were made by Loeb and by Harrop and their respective co-workers (214,216,219,220,362). Following adrenalectomy or cessation of maintenance therapy with cortical extract, the blood showed a fall in total base and total acid. Similarly the sodium, chloride, and bicarbonate were decreased, whereas potassium and NPN were increased. In balance experiments a marked loss of sodium, chloride, and increase in total base was found in the urine. Potassium and total nitrogen were decreased. Such changes were not due to vomiting or diarrhea. The urine volume was increased, but despite this the sodium excretion in the urine was so great that the concentration was increased. The relative loss of sodium, therefore, was greater than the loss of water. Chloride loss paralleled that of sodium, but was not necessarily equivalent (219,362,516,579). When adequate treatment with cortical extract was instituted, the altered electrolyte pattern of adrenal insufficiency rapidly returned to normal and the marked renal wastage of sodium and chloride was prevented. The extent of the changes in various electrolytes found after cessation of treatment in adrenalectomized dogs, and the restorative effect of treatment has been listed in the tables in Section IV,A showing the biochemical changes following adrenalectomy.

As previously noted, it has been found that the adrenalectomized animal can be kept in normal condition by the administration of sodium salts without the use of extract. Sodium chloride was initially used, either mixed with the diet, given by stomach tube, or placed in the drinking water. If adrenalectomized dogs are given 7-8 g. sodium chloride daily they appear normal at first, but usually within 2 weeks they have lost weight and refuse to eat. At this time the bicarbonate of the blood may be as low as 29 volumes per cent, and the blood sugar at hypoglycemic levels. Blood urea, sodium, and chloride are at a normal levels. At this point the injection of sodium bicarbonate and glucose restores the animal to normal. For prolonged maintenance treatment the addition of 3 g. sodium bicarbonate to the salt allowed the animal to survive for prolonged periods, gain weight, and exhibit normal biochemical findings. Allers (3) found the substitution of sodium citrate for sodium bicarbonate to allow even greater improvement. Harrop *et al.* (220), in replacement experiments with sodium lactate, concluded that the anorexia following treatment with salt

alone was related to low plasma chloride (90 meq./l. or 315 mg. %). This apparently caused a diminished secretion of gastric juice and absence of free acid from the stomach secretion. A balance between sodium and chloride, to allow for the proportionately greater urinary secretion of sodium over chloride, was most easily achieved by using a mixture of sodium chloride and bicarbonate. Adrenalectomized dogs were maintained for as long as 5 months without symptoms on such a salt mixture (220). An additional factor which should be calculated in the diet for such experiments is potassium. Allers and Kendall (4) found a daily intake of less than 200 mg. potassium in the diet was practical and allowed the replacement therapy of sodium and chloride to give optimal results. Detailed observations on such treatment, and of the type of crisis which may occur in dogs, are described (74,430). The optimal salt content of the diet for the adrenalectomized animal is therefore one containing both sodium and chloride, but the former should be proportionally greater. The potassium content should be as low as possible. That beneficial effects of various salts and sodium chloride could also be obtained in the treatment of adrenalectomized rats was shown by Rubin and Krick (515,516) and Gaunt, Tobin, and Gaunt (183). More recently, it has been found that the optimal daily intake of sodium chloride for an adult adrenalectomized rat was from 650 to 940 mg. A level of 339 mg. was not sufficient for growth or survival, whereas 1200 mg. daily was injurious (12).

Anderson and Joseph (10) studied the excretion of radioactive sodium and potassium in adrenalectomized rats. Adrenal insufficiency caused a more complete and more rapid elimination of  $\text{Na}^{24}$ , whereas the reverse was true for  $\text{K}^{42}$ . The drinking of 1% sodium chloride solution restored the electrolyte changes to a normal pattern (11). It is of interest that Richter has found that adrenalectomized rats will select and drink exclusively fluid containing sodium salts, and so maintain their existence. Treatment with cortical extract or DCA decreased their appetite for salt, but caused an increased intake of dextrose solution (490,492). Normal rats treated with DCA also showed an increased salt appetite (487).

Adrenalectomy has also been found to exert a definite action on the intestine so that the rate of absorption of electrolytes is retarded. This is true for both the rat (68,595) and the dog (108). However, evidence has been presented from balance studies in the adrenalectomized rat that reduction of chloride in the feces occurs and tends to compensate for the increased excretion in urine (108,529). This change reverts to normal after treatment with cortical extract or DCA.

Cases of Addison's disease may be successfully treated and the normal blood chemistry maintained by the use of sodium salts (358,592). Harrop *et al.* (224) and Loeb (359) have successfully used the withdrawal of salt

from the diet as a provocative test in cases of suspected adrenal insufficiency. Such a procedure invokes the characteristic blood findings and symptoms of acute adrenal insufficiency in a few days' time, but obviously requires constant supervision and the rapid institution of adequate extract and salt therapy on completion of the test. Although treatment of cortical insufficiency can be accomplished by the administration of sodium and chloride and restriction of potassium, it should be emphasized that such replacement therapy is only partial. In such a case withdrawal of food is followed by hypoglycemia and a failure of carbohydrate and protein mechanisms, which are to be discussed later. Similarly, the lowered general resistance to infections, toxins, and environmental stress is not restored to normal by salt therapy.

The defect in sodium metabolism is probably not the cause of death of the adrenalectomized animal, although in early observations it had been considered a probability. Although the loss of salt and water by the kidney and the resulting dehydration undoubtedly contribute to the circulatory changes and final collapse of the adrenalectomized animal, it has been found that the symptoms of adrenal insufficiency and degree of sodium depletion do not necessarily run parallel; also circulatory collapse in adrenal-deficient dogs may occur without any changes in blood sodium or body water (613). An animal in acute insufficiency may be revived by the intravenous injection of hypertonic saline, which restores the circulation and electrolytes. This effect on the circulation is, however, only temporary, and the animal dies within a few hours with typical hypotension. At this time, however, the serum electrolytes may still be found at normal or above normal levels (608,612). Cases of Addison's disease have been reported by Loeb (360) in which death occurred with essentially normal blood values for electrolytes. Certain species such as the marmot and the opossum show an increase in blood sodium and chloride and do not excrete increased amounts of sodium after adrenalectomy. They may die of insufficiency despite the absence of the usual effect on electrolytes, although following a two-stage operation survival is often prolonged (47, 244, 578). In the converse state it has been shown that if adrenalectomized dogs are kept on a salt-free diet they become depleted of electrolytes and maintain a low plasma sodium level. Such animals, however, can be maintained in normal health or revived from circulatory collapse following extract withdrawal by cortical-extract therapy without changing the electrolyte levels (609).

The evidence that alterations in sodium balance may be differentiated from other changes due to adrenal insufficiency has suggested that this function may be separately and specifically controlled. Hartman and workers in his department have shown over a number of years that a separate so-

dium factor is present in cortical extracts and that it possesses some interesting properties. Originally it was found by Hartman, Lewis, and Toby (237,238) that repeated intravenous injections of cortical extract in normal dogs and in man led to a decreasing response in the retention of sodium. Later, Hartman and Spoor described methods for fractionating cortical extract into a "Na factor," and a factor for life maintenance "cortin," the name originally designated by Hartman for his adrenal extract (243,628b). The "Na factor" had the ability to keep adrenalectomized cats alive for considerable periods, although their reactions were not normal. The plasma sodium level was directly related to the dosage used in treatment. "Cortin" caused a potentiation in effect of the "Na factor." The blood urea was somewhat increased during treatment. No effect was observed on plasma potassium or blood sugar (234). Following repeated intravenous injections the sodium-retaining action in normal animals was gradually lost. This refractivity did not develop if injections were given subcutaneously or if an adrenal extract of the same species was used intravenously. The serum from an animal made refractory to the "Na factor" was active when transferred to a normal animal. The active substance apparently was contained in the pseudoglobulin fraction of the serum. The reaction appeared to be of an immunity type, the "Na factor" being combined normally with some protein. When refractivity developed in an adrenalectomized animal, the plasma sodium level was reduced to that usually associated with insufficiency, yet the animal's condition did not markedly change. Such refractivity did not develop after injections of corticosterone or DCA (232,233,240,654). "Cortin" devoid of the "Na factor" kept adrenalectomized animals alive and in good condition indefinitely. The plasma sodium of these animals, however, was at the low level typical of insufficiency (234).

The primary organ affected by adrenal insufficiency with regard to sodium metabolism is undoubtedly the kidney. It seems likely that in the absence of the adrenal hormone the kidney tubule cells are unable to reabsorb adequately the sodium from the glomerular filtrate, even though the plasma sodium level is steadily falling. At the same time the tubular cells fail to excrete potassium, phosphate, etc. when the level of these ions is rising in the plasma. As a secondary result from the loss of sodium and chloride, dehydration, and circulatory failure, there is a diminished rate of glomerular filtration (211).

### *1. Effect of Adrenal Hormones*

Following the initial demonstration of the importance of electrolytes in patients and animals with cortical insufficiency, a large number of balance studies of the biochemical changes induced by cortical extract were made.

These at first concerned patients with Addison's disease (636,643,644) or adrenalectomized animals (219,636). However, it was soon shown by Thorn, Garbutt, Hitchcock, and Hartman (630,642,643) that the normal human and the normal animal (221,238) also showed similar changes after injection of cortical extract (*cf.* 332a).

In a study on eighteen normal dogs the following renal excretion changes occurred after the administration of 20-40 cat units of cortical extract: Sodium retention was 42-89%, chloride retention 10-58% (238). Such observations on renal excretion led to the development of a method of assay for cortical hormones. Harrop and Thorn (221) proposed that normal dogs be used for assay. These were kept on a constant dietary regimen and sodium intake. The urine was collected by catheterization at various intervals over 24 hours following extract administration. Water and salt were given by stomach tube. Control values obtained for individual animals showed that the maximum variation of excreted sodium was less than 5 meq. daily. Tests could be repeated after 3-day intervals. Using such a method the typical retention of sodium and water and excretion of potassium induced by cortical extract was found. The minimum amount of extract necessary to produce a 15-20% reduction in the 24-hour renal excretion of sodium was used as a standard. Repeated doses of extract were more effective than the same amount given as a single injection, whereas the oral activity was very slight. Retention of sodium was also found to take place when the animals were on a potassium-free diet. Hartman and Spoor (243) found it unnecessary to collect urine for more than 6 hours to obtain accurate values. They kept their animals on a standard diet with added sodium chloride. Following initial catheterization to empty the bladder, 100 ml. water was given by stomach tube. The bladder was emptied again after 6 hours. The extract must be given subcutaneously to prevent refractiveness to repeated injections. A unit was defined as one-tenth the amount of extract required to give 30% retention of sodium. In a later paper desoxycorticosterone was used as a standard (235). The dose was adjusted so that it caused not less than 35% or more than 65% retention of urinary sodium over the animal's control value (usually 0.6 to 0.8 mg. for a 10-12 kg. dog). The dose of unknown substance was adjusted until an equivalent response was obtained. The final unit for sodium retention was defined as one-tenth of that amount of material which will cause the same sodium retention as 0.7 mg. desoxycorticosterone. Two commercial cortical extracts contained 1.4 to 2.2 units/ml.

From comparative assays which have been reported it would seem that desoxycorticosterone and DCA are the most active chemically pure substances affecting sodium retention. Corticosterone and 11-dehydrocorticosterone are about half as active. Cortical extract is also very active.

Values given in several reports using normal dogs (243, 338, 637, 638, 640) have been calculated and are given in Table XIII. It will be noted that adrenal steroids are not the only ones possessing the action on sodium metabolism, since substances like  $\alpha$ -estradiol and progesterone are also active in this type of test (333,646). Thorn, Engel, and Lewis (640) and others (154e,308) have found in short-term experiments that Compound

TABLE XIII

Substance <sup>a</sup>	Ref.	Dose, mg.	% Na retention	% K excretion
Desoxycorticosterone	243	0.5	17	...
	243	1.0	32	...
	243	2.0	56	...
	243	4.0	68	...
	640	1.0	48	...
DCA	638	1.0	31	27
Corticosterone	243	4.0	28	
	638	4.0	15.5	19.5
	640	4.0	14.5	...
Cortical extract "purified"	638	10.0	40	30
	638	2.0	22	..
Androstenediol	637	40.0	Inactive	..
Androstenedione	637	40.0	Inactive	..
Cholesterol	637	200	Inactive	..
Progesterone	637	20	25	..
Estradiol	637	5	41	..
Testosterone propionate	637	125	7	..
17-Hydroxycorticosterone	640	1	% Na Excretion	
	640	5	0	
	640	8	27	
	640	25	38	
Compound E	640	...	150	
	640	6	380 (adrenalectomized dogs)	
	640	25 cc.	75 (normal rats)	
Adrenaline	640	1:250,000	22	108

<sup>a</sup> Dehydrocorticosterone "about same as corticosterone" (638).

E and 17-hydroxycorticosterone have an opposite action, and cause a marked excretion of sodium in the normal or adrenalectomized animal. Adrenaline has a similar action. Data on this type of effect is shown in Table XIII. It has been suggested that the influence of these compounds which are contained in cortical extract may explain why marked sodium retention, increased plasma volume, and hypertension do not follow prolonged administration of cortical extract in contradistinction to the action of DCA (640). Dorfman, Potts, and Feil (111) have recently devised a

sensitive method for the assay of desoxycorticosterone. Using radiosodium,  $\text{Na}^{24}$ , they have demonstrated a significant retention in adrenalectomized rats after a dose of only 0.98  $\mu\text{g}$ . DCA. The opossum apparently is refractory to the action of sodium-retaining substances, although DCA has some effect (585). In cases of Addison's disease DCA therapy has been shown to be followed by a marked retention of sodium, chloride, and water and the development of hypertension. In such cases considerable danger is present, especially if the diet contains excessive sodium or is deficient in potassium. In the latter case sodium is more readily retained and the replacement of potassium in the muscles by sodium takes place (338, 655). The action of 11-dehydrocorticosterone on salt retention in Addison's disease is much less marked than that of DCA (154d, 449b).

### C. POTASSIUM METABOLISM

Definite changes in the blood level of potassium following adrenalectomy were noted by Baumann and Kurland in their report in 1927 (20). Figures were obtained for twelve adrenalectomized cats; the average plasma potassium was 28 milligrams per cent as compared to a control value of 19 milligrams per cent. Hastings and Comper (247) later found a marked increase in serum potassium following adrenalectomy in dogs. The increase was as great as 50% at the end of 48 hours, and reached a level of 20 meq. at death, compared to a normal value of 3 meq. Generally it is found that the serum potassium behaves in the opposite manner to sodium in adrenal insufficiency, as if to attempt to conserve the blood electrolytes. Serum magnesium is also usually increased although calcium is probably only slightly affected. In balance experiments potassium is retained by the cortical-deficient animal and the level in the urine is reduced; cortical extract, DCA, or a high sodium chloride intake results in a return to normal of blood and urine through elimination of potassium by the kidneys.

Shortly after the value of a high sodium and chloride intake was established it was shown that a low potassium intake was also necessary to maintain adrenalectomized animals (4,5,430) or cases of Addison's disease (525, 692). In the adrenalectomized dog it was also found that conversely the addition of 0.5 g. potassium daily in the diet sent the dog into a form of crisis (5). Zwemer and collaborators have made extensive observations on potassium and its metabolism in adrenalectomized animals. In such cases it has been found that the tolerance to potassium is greatly reduced (658,728). When adrenalectomized rats were fed a diet containing 0.5% potassium a high mortality was found, but this was markedly reduced when the potassium content used was only 0.1%. Adrenalectomized rats die rapidly, however, from drinking water containing 0.1% potassium chloride (179). Adrenocortical transplants partially protect adrenalectomized rats

against toxic doses of potassium (659). Adrenalectomized cats would not voluntarily continue to eat a diet high in potassium, whereas normal cats ate it readily. (The same appears to be true in the case of dogs, 430.) When potassium chloride was injected intraperitoneally in a dose of 200 mg./kg., a normal animal showed a slight transitory rise of serum potassium to a maximum of 30 milligrams per cent, but this had returned to normal in 2 hours. In contrast, an adrenalectomized animal showed serum values of 40 to 60 milligrams per cent, which remained high until death took place in about 7 hours. The symptoms before death resembled those of acute cortical insufficiency. In adrenalectomized dogs it has been shown that, when potassium salts are injected, symptoms commence at approximately the same serum level of potassium as they do in intact dogs similarly treated. However, only one-third as much injected potassium was needed in the adrenalectomized dog to reach a toxic level (700). The tolerance of the adrenalectomized dog to intravenous potassium chloride is reduced below normal so that a dose of 15 mg./kg. is usually fatal, but 10 mg./kg. is tolerated. Blood values are found to be markedly increased when potassium tolerance curves are made on adrenalectomised rats (394). When a crisis is precipitated in an adrenalectomized dog by potassium injections, the serum level may be at 35-40 milligrams per cent. The blood urea at this time may be of a normal value and therefore does not serve as an index of the animal's condition. Cortical-extract injections or adequate salt intake will rapidly restore such an animal to a normal condition (430). The toxic effects of potassium in the adrenalectomized animal may still be elicited, even though the intake of salt is quite high (5). The quantitative relations of sodium and potassium intake in the diet to the retention and excretion of those elements have been studied and reviewed by Harrop (215). The feeding of potassium to an adrenalectomized dog maintained in balance by salt and cortical extract led to an increased loss of sodium in the urine with simultaneous retention of potassium. Eventually, symptoms of typical adrenal insufficiency developed. Conversely, increasing the intake of sodium chloride in a deficient animal tended to reduce the potassium level of the blood and increase the urinary output. The animal which is not receiving any form of extract treatment is much more sensitive to changes in either sodium or potassium intake.

The toxic manifestations following potassium administration in adrenal insufficiency have been suggested as the basis for a provocative test in suspected cases of Addison's disease (98,727). A solution of potassium salts was administered by mouth in a dose of 10 mg. potassium/pound body weight. Such a dose does not cause an increase in plasma potassium in a normal individual, although double the dose gives a brief rise of 10 milligrams per cent. In an Addisonian, however, this smaller dose causes a

marked rise of plasma potassium of from 20 to 60 milligrams per cent in a half hour's time. It is suggested that the method is more rapid and relatively safer than that involving sodium depletion. Cortical-extract administration is followed within 15 minutes by a reduction of plasma potassium and the alleviation of any untoward symptoms (727).

Although potassium administration to adrenalectomized animals in acute experiments is followed by symptoms resembling those of adrenal insufficiency, there is little evidence that potassium accumulation *per se* is the cause of death in adrenal insufficiency. In experimental animals there is not necessarily a close relationship between the blood level of potassium and the onset or severity of the symptoms of adrenal insufficiency (207, 430,483,613). In Addison's disease crisis may be present with little potassium retention, or conversely high serum potassium levels may occur when the individual is symptom-free (361). Similarly, attempts to produce comparable toxic symptoms by potassium in humans (325) and in normal dogs (533a) have not always been successful.

Like the changes found in sodium and chloride, those concerning potassium appear to be primarily related to a defect in kidney function caused by the lack of cortical hormones. At the same time, however, there is probably a breakdown in the mechanism regulating potassium equilibrium between the tissues and blood plasma (394). The studies of Harrop and collaborators clearly showed that the increase in plasma concentrations of potassium following adrenalectomy were due to the decreased capacity of the kidney to excrete potassium. Correction of such a defect occurred following cortical-extract injections (214,219). Harrison and Darrow (211) believe that the disturbance appears to be in kidney tubule function, so that potassium and phosphorus are reabsorbed to a much greater extent than normal by the adrenalectomized animal. The volume of urine excreted is also of importance, since factors tending to increase the rate of urine excretion would also increase the excretion of potassium (and also of sodium). The administration of sodium salts, causing an increased secretion of urine would therefore cause an increased excretion of potassium.

### *1. Effect of Adrenal Hormones*

As previously noted, adrenocortical extract corrects the altered potassium metabolism of the adrenalectomized animal, the increased blood levels being restored to normal at the same time as the urinary output is increased. Cortical extract also enables normal animals to withstand larger amounts of injected potassium. Thus, the mortality in both guinea pigs and mice from a dose of potassium usually toxic may be reduced by pre-treatment with cortical extract (728). In immature mice it was found

that the LD<sub>50</sub> for injected potassium chloride was 6.65 mg./10 g. body weight. Adrenalectomized mice were more sensitive so that a dose of 5.75 mg. caused the same mortality. When normal mice received 1 mg. DCA it required a dose of 7.7 mg. potassium chloride to cause 50% mortality. Similarly, the tolerance of adrenalectomized mice could be raised above normal by either DCA or cortical extract. These observations led to the suggestion that such mice could be used as a basis for a method of assay for DCA (657).

DCA has a marked effect on potassium metabolism as well as causing a retention of sodium, chloride, and water, in the adrenalectomized animal. The prolonged administration of 25 mg. daily to normal dogs was first shown by Kuhlman, Ragan, Ferrebee, Atchley, and Loeb (338) to result in a marked lowering of serum potassium by approximately 45%. The urine volume was markedly increased. At the time of these findings all the dogs showed attacks of periodic weakness with inability to raise the head or to stand. The administration of potassium salts was found to alleviate and protect the animals against such attacks. Similarly, withdrawal of DCA therapy was followed by rapid recovery. The replacement of muscle potassium by sodium is responsible for the paralysis (147). The use of DCA therapy in Addison's disease may give rise to serious consequences if the accompanying diet is low in potassium, as retention of sodium and water is much more pronounced (191,655). The action of DCA may be on the kidneys or cell permeability. Talbott *et al.* (625) found that the kidney clearance of potassium was not normal in patients with Addison's disease maintained on DCA therapy. As previously noted, in acute experiments large doses of corticosterone or 17-hydroxycorticosterone cause increased urinary excretion of potassium and nitrogen, and also of sodium and chloride (308,640).

#### D. INTRACELLULAR FLUID AND ELECTROLYTES

Balance studies on untreated adrenalectomized dogs, or after cessation of maintenance extract therapy, have shown that the increased excretion of urinary sodium was accompanied by a moderate diuresis (219,362). It was suggested that more fundamental changes took place in the distribution of fluid and electrolytes between the blood and tissues, and that the adrenals were an important factor in the mechanism which regulated the internal-fluid distribution in the body (609,611,619). Later observations by a number of workers have shown that in adrenal insufficiency muscle and other tissues show an increased water content. This and the increased urinary volume, associated with the renal wastage of sodium and chloride, combine to increase blood concentration. The increased fluid in the muscle and liver of the adrenalectomized rat was shown by Silvette and Britton

in 1933 (46,579). Treatment with cortical extract corrected this change. In adrenalectomized cats the increased water content of muscle was more pronounced as insufficiency increased. Chlorides of serum, liver, and muscle were reduced (577).

Muntwyler, Mellors, and Mautz (421) have made extensive analytical determinations on muscle, comparing 24 normal and 7 adrenalectomized dogs. Their averaged results have been summarized in the following table (values are given per kg. fat-free muscle):

Measurement	Normal	Adrenalect.
Cl, m.eq.....	19.5	15.1
Na, m.eq.....	28.2	16.8
K, m.eq.....	83.5	87.8
H <sub>2</sub> O, g.....	761.4	776.4
Extracellular H <sub>2</sub> O, g.....	159	137
Intracellular H <sub>2</sub> O, g.....	604	641
Collagen N, g.....	4.88	6.0

The lowering of muscle sodium and chloride and increase of potassium following adrenalectomy are clearly shown. The noted increase in water content was due to an increase in intracellular water. Other workers have reported similar findings in dogs (103). The changes in the dog are similar to those originally described in the cat by Hegnauer and Robinson (252). With the cessation of maintenance therapy in this species the muscle potassium increased, whereas the water content increased or remained constant, the increase being due to intracellular water. The red blood cells of the cat behave in a similar manner and the results are given in Table IX. The increase in potassium in the red blood cells of adrenalectomized dogs just before crisis and a reversal of effect by cortical extract have also been reported (430).

Extensive studies on the cat have also been made by Darrow, Harrison, and Tafel (103). Whereas they found a significant increase in the muscle potassium and intracellular water after adrenalectomy, the alterations in chloride, sodium, phosphorus, and protein were slight. Changes in other tissues, such as the heart, liver, and kidneys, tended to be of smaller magnitude and less constant.

The adrenalectomized rat has been studied by Harrison and Darrow (212), but they found no consistent alteration in muscle water content. Sodium and chloride were reduced and potassium increased. Potassium in liver, on the other hand, was not increased. Restoration to normal of all constituents followed cortical extract or intraperitoneal injections of hypertonic solutions of sodium chloride and bicarbonate. Treatment of

the adrenalectomized rat by implantation of DCA pellets has also been found to reverse the changes of adrenal insufficiency. The results of such therapy and of sodium chloride are shown in the following table (58):

Meq./100 g. dry, fat-free muscle	Untreated		D.C.A. pellets		NaCl
	Normal	Adrenalect.	Normal	Adrenalect.	Adrenalect.
H <sub>2</sub> O, g. % . . .	75	76.0	74.3	75.8	75.2
Na . . . . .	10.0	8.9	14.1	15.6	9.5
K . . . . .	32.0	118.1	30.2	29.9	36.1
Ca.. . . . .	1.4	1.4	1.1	1.4	1.2
Mg. . . . .	8.3	5.9	7.8	6.8	7.1

Observations have been made on isolated muscle removed from adrenalectomized rats. In one case it was shown that water was taken up more rapidly in a hypotonic solution by such tissue when compared to that of normal animals. Conversely, water was lost more rapidly in hypertonic solutions (701). These results indicate a change in permeability of the muscle cells but have not been confirmed (461). In the adrenalectomized toad the sodium in skeletal muscle, but not in the liver, is markedly decreased (67).

The restoration of the altered muscle constituents of the adrenalectomized animal to normal by cortical extracts may take as long as 48 hours of treatment. On the other hand, the symptomatic improvement of an animal in crisis on such therapy is marked and rapid (212,421). This would suggest, therefore, that the disturbances of adrenal insufficiency are not directly due to the alterations described in the tissues.

The same changes as those found in the tissues in adrenal insufficiency may also occur under other conditions, and it is questionable, therefore, whether the action of adrenal hormones is a direct one on membrane permeability or that the tissue changes are secondary to alterations in plasma constituents following abnormal kidney regulation. Thus a lowering of extracellular electrolytes under various conditions in the intact animal leads to an increased water content of muscle and red blood cells (104,129,404, 421,713).

Nephrectomized rats, as the plasma level of potassium increases, show an increased intracellular potassium similar to that seen in adrenalectomized animals (103). The muscles of normal rats gain sodium and lose potassium when they are fed a potassium-free diet (254) and the injection of potassium will lower muscle sodium and cause an increase in potassium (408). Experiments in which isotonic 5% glucose was injected intraperitoneally and subsequently recovered induced a depletion of extracellular sodium. After such treatment symptoms resembling adrenal insufficiency

are produced in the intact animal (104,534). The blood serum sodium is reduced, hemoconcentration is found, and there is a lowered resistance to stress (185,212,421,496). The red blood cells lose sodium into the plasma (496). The adrenalectomized animal is extremely sensitive to intraperitoneal injections of glucose, death usually following such a procedure, which is well tolerated by the intact animal (181,214,480,608,706,711). The changes in blood serum in the adrenalectomized animal are exaggerated by such treatment (480). Cortical extract or DCA will reduce the effects of intraperitoneal glucose (481).

Certain experiments have been put forward as indicating that the adrenal may directly influence the entrance of potassium into muscle. In adrenalectomized animals injected potassium is transferred less rapidly from the blood stream into the tissues than in normal animals (394,700). In one experiment on adrenalectomized nephrectomized rats, cortical extract was found to retard the rise in potassium concentration and prolong life (305), although other workers have not found such clear-cut changes (230,387).

The behavior of muscle potassium during contraction has been studied in the adrenalectomized animal. Somogyi and Verzar (587) have reported that, whereas potassium was liberated from the muscles of normal cats during contraction elicited by nerve stimulation, it was not released in adrenalectomized cats under identical conditions. Jordan and others (319, 656a), however, have not confirmed this work in rats and cats and find that muscle potassium behaves in an essentially normal manner after muscle stimulation even in adrenal insufficiency. DCA undoubtedly has a profound action on muscle potassium, either directly or indirectly. It will prevent the increase in intracellular potassium following adrenalectomy and will reduce tissue potassium in normal animals (58,147,407). Prolonged treatment causes loss of potassium from the muscle with replacement by sodium, giving rise to symptoms of muscular paralysis (147,338). The effect is more rapid and severe if the animal receives a low-potassium diet (102). Such paralytic effects do not follow treatment with cortical extract. In DCA-treated rats a reduction of potassium of approximately 20% takes place in the brain tissue (718). A comparable effect was believed to be related to the reported effectiveness of this form of therapy in a case of epilepsy (392).

#### E. WATER EXCRETION

Associated with the changes which occur after adrenalectomy in blood and tissue electrolytes and water are variations in the urine output. Many of the early workers (223,362,618) noted an increase in urine output after adrenalectomy associated with increased over-all excretion of sodium and chloride, although the electrolyte concentration might be reduced in the

urine. The diuresis, however, was not sufficient to explain the total reduction of extracellular fluid found in the depleted animal, or to explain the symptoms which develop. The diuresis following adrenalectomy may be clearly demonstrated in the rat, where the urine output may be increased four to five times (21,181,528,529). The diuresis continues as long as the animals are maintained in good condition by diet, and the addition of salt increases the urinary volume. When the animals show signs of insufficiency the urine output falls below normal and a terminal anuria may occur. The water intake of rats showing a diuresis tends to remain constant and does not increase proportionally to the raised urine volume.

Despite the diuresis following adrenalectomy the kidney rapidly loses its ability to excrete water administered to the animal. As a result, if water is given in repeated doses, the adrenalectomized animal cannot excrete enough urine and the typical symptoms of water intoxication, as described by Rowntree (513), ensue. Working with adrenalectomized mice, Rigler in 1935 (494) first found that they were sensitive to water intoxication. Swingle, Parkins, Taylor, and Hays (610) showed that adrenalectomized dogs were similarly sensitive to water. If normal animals were given 25 ml. water/kg. body weight by stomach tube at hourly intervals until the onset of convulsions, they would then recover spontaneously. On the other hand, adrenalectomized animals treated in the same way would subsequently die unless treated by intravenous cortical extract or hypertonic sodium chloride. The administered water was found in the adrenalectomized dogs to cause a drop in serum sodium from 139 to 117 and in chloride from 107 to 82 meq./l. Potassium rose slightly and the blood became more concentrated. In further studies (621) it was found that 50% of normal dogs show symptoms of water intoxication after ten hourly doses of water, the typical changes being progressive excitability, salivation, severe convulsions with opisthotonus. These occur when the amount of water retained by the animal is 140 ml./kg., which is equivalent to an intracellular hydration of 121%. In the adrenalectomized dog convulsions follow after some six doses, as little or no urine is excreted. An increased susceptibility to hydration is apparent, since the intracellular hydration only equaled 115%. Pretreatment with either three injections of 5-10 mg. DCA or 3 ml. cortical extract/kg. prevented the increased sensitivity to water intoxication and allowed urine excretion to become normal. Adrenalectomized dogs on a high salt, low potassium diet have normal urinary excretion responses to water except in hot weather (337).

In humans there apparently is the same defect in water excretion when adrenal insufficiency occurs (149,473,497). The urinary response to the ingestion of 20 ml. water/kg. body weight has been used as a diagnostic point in Addison's disease. In such cases the urine does not become diluted

and the urinary volume in 4 hours is extremely low. This test has been described by Robinson, Power, and Kepler (497) and the subject reviewed (172a,349a).

Similar findings have been described in adrenalectomized rats, since Silvette and Britton in 1933 found that the excretion of injected water was defective (128,249,572,579,596). Gaunt and collaborators have found that, whereas normal rats would withstand the oral administration of 6 ml. water/kg./hour for 6 hours without symptoms, the adrenalectomized (or hypophysectomized) rat showed collapse and convulsions in 4 hours and frequently died in 24 hours after the same treatment. The normal rat's

TABLE XIV

Substance	Dose for protection against H <sub>2</sub> O intoxication	Comparative assay
Cortical extract in oil	0.8 ml./day	0.05-0.1 ml./day for 80% survival and growth—adrenalect. rat.
Aqueous cortical extract	1.0 ml./day	1.0 ml./day (= 75 g. fresh glands) for maintenance—adrenalect. dog.
Amorphous fraction	1.0 ml./day ineffective 2.0 ml./day effective, but urine excretion not completely restored.	1.0 ml./day for maintenance—adrenalect. dog.
DCA	3.0 mg./day	0.05 mg./day for 80% survival and growth—adrenalect. rat.
Compound E	1.0 mg./day	

urine output in 8 hours was 85% of the volume of water given, whereas little urine was secreted by adrenalectomized animals. Cortical extract protected the animals and allowed urine excretion to become normal (172,181,249). Eversole, Gaunt, and Kendall (146) have compared the effectiveness of adrenal derivatives in protecting adrenalectomized rats against death from water intoxication, and in restoring the defective urinary response. The results have been summarized in Table XIV. It is of interest to note that the amount of cortical extract in oil to protect against water intoxication is ten times that required daily for life maintenance of the adrenalectomized rat. DCA is similarly even less effective,

since some sixty times the dose is required to prevent water intoxication. The amorphous fraction was effective, but Compound E was very effective—at least three times more so than DCA. In other experiments it has been found that estrone, progesterone, and testosterone are ineffective in preventing water intoxication when compared to cortical extract (178).

The prolonged administration of DCA to normal dogs has been shown to lead to marked polyuria and polydipsia by Kuhlman, Ragan, Ferrebee, Atchley, and Loeb (338). In initial experiments 25 mg. DCA was injected subcutaneously daily and the urine volume increased approximately 2.5 times. The water intake was similarly raised. The addition of sodium chloride to the diet augmented the fluid exchange so that urine volumes exceeded 2500 ml. daily. Although the condition produced resembled diabetes insipidus, it was believed to differ because Pitressin in large doses did not markedly reduce the fluid exchange, and restriction of fluid intake did not lead to a negative fluid balance and dehydration. Animals treated with DCA in this manner exhibited attacks of paralysis attributable to replacement of potassium by sodium in the muscles, secondary to the urinary retention of sodium and the loss of potassium, as described previously (470). Polyuria and polydipsia can also be produced in dogs by a daily dosage of DCA of 2 or 4 mg. and paralysis does not develop (418). The increased intake of water apparently precedes the polyuria and the amount of sodium chloride ingested has a direct bearing on the volume of urine excreted. In such experiments it was found that both large doses of Pitressin in oil and water deprivation caused suppression of the polyuria. The condition produced by this dose of DCA was therefore believed to resemble true diabetes insipidus.

Similar changes in fluid exchange have been described following DCA treatment of rats. These changes may occur in normal, adrenalectomized, or hypophysectomized animals (93,470,541,542,560). At the same time retention of sodium and chloride occurs, and in some cases Pitressin has controlled the increased urinary output (93). The mechanism of the changes produced by DCA in fluid exchange is not clear, since it may be secondary to altered electrolyte metabolism (620) or more directly concerned with the complex hormonal control of urine secretion (93,541). From a study of the protective effect of DCA against water intoxication in normal and adrenalectomized nephrectomised rats, Birnie, Eversole, and Gaunt have concluded that an extra-renal action may be involved (31a).

## VI. Adrenal Influence on Heart, Arterioles, and Capillaries

### A. HEART

One of the most perplexing problems concerning adrenal insufficiency is the mechanism of the cause of death. Thus, alterations in sodium,

potassium, water balance, carbohydrate metabolism have been considered. In all cases, however, the changes do not appear of great enough magnitude and the same changes can be produced to the same extent by other means without causing death. While it would appear that the combined alterations of the factors above may have an additive effect, there is considerable evidence to show that cardiac failure is probably the immediate cause of death in adrenal insufficiency. That changes occur in the heart rate and blood pressure in chronic adrenal failure was commented upon by a number of the earlier workers. Rogoff and Stewart (501) noted a slowing of the heart rate and the extent of the bradycardia was noted by others (219, 430, 617). Hall and Cleghorn (207) in a detailed study found that with increasing adrenal insufficiency in the dog there was associated an irregular apex beat and the rather sudden onset of bradycardia. Extra systoles were present and electrocardiograph tracings showed changing T-waves, and at times complete absence of P-waves. The bradycardia was often extreme, a rate of 35-45 beats per minute being found instead of the normal 110-120. Evidence of coronary spasm and cardiac ischemia was indicated by heart block and auricular fibrillation. In the later stages of insufficiency no acceleration in heart rate could be produced by atropine or section of the vagi. Even the most severe changes were found to disappear rapidly if the dog was given intravenous hypertonic glucose and saline, cortical extract, or both. In control experiments it was shown that the level of potassium in the blood (30-40 mg./100 ml.) which occurred in adrenal insufficiency was not enough to produce similar changes when induced in normal dogs. Cardiovascular effects in normal animals were not evoked until the level of blood potassium was raised to 50-60 milligrams per cent. Other workers, however, believe the cardiovascular changes are related to an increase in potassium (430, 700, 728). Nicholson and Soffer (429) found a striking correlation between plasma potassium levels of adrenalectomized dogs and auricular fibrillation—a plasma level of 52 milligrams per cent being followed by fibrillation. The hearts of animals showing evidence of cardiac damage following adrenalectomy were found to show coronary and myocardial lesions at autopsy (207). Cardiac irregularities in adrenal insufficiency in the dog have been found to occur before the blood pressure had fallen below 80 mm. and could be corrected immediately following the intravenous injection of DCA dissolved in propylene glycol (76). It is of interest to note that in other experiments the ability of DCA to prevent circulatory collapse due to trauma seemed dependent on the use of cortical extract for the preliminary maintenance treatment (483).

#### B. BLOOD PRESSURE AND PLASMA VOLUME

Hypotension was one of the striking features noted in Addison's disease, and it is also one of the most constant signs found after experimental re-

moval of the adrenals. In the dog the arterial pressure slowly and progressively falls from the time replacement therapy is discontinued. The terminal collapse of the animal is always associated with extremely low blood pressure levels. The reduced blood pressure can only be adequately restored and maintained by treatment with cortical extract or hormones (346,617,619), although a high salt intake alone may cause a raised blood pressure in some cases of Addison's disease (361). The pressor action of cortical extracts upon the low blood pressure of dogs in extreme insufficiency has been shown to be independent of changes occurring in serum electrolytes, hemoconcentration, or fluid distribution (447). The changes in blood pressure and blood urea apparently occur in an inverse ratio (619). The increased urea occurring with the fall in blood pressure rather than before it as previously claimed (223,399). Cortical extracts, even in excessive doses, will not cause persistent or even transitory hypertension in animals or man (38,203,607). On the other hand, DCA has been found to cause an abnormally high blood pressure in rats (38,203), dogs (338, 344,500,607,647), and in humans, especially when the salt intake is high (148,381,449,524,586,631,634). The pathological changes produced by overdosage with DCA in experimental animals will be described later. Hypertension in rats has also been noted to follow the administration of large doses of testosterone, progesterone, estradiol, and diethylstilbestrol, possibly through an injurious effect on the kidney with the resultant liberation of renin (203).

Closely paralleling the changes in blood pressure are alterations in plasma volume. The low plasma volume in severe adrenal insufficiency has frequently been noted in man (363) and animals (72a,223,250,326,617,622, 709). Treatment with cortical extract will restore the plasma volume to normal, but will not raise it above normal levels. DCA, however, also has the ability to restore the lowered plasma volume to normal but will increase it to abnormally high levels in man and animals, irrespective of whether they have functioning adrenals (82,83,148,363,524,607,648). A daily dose of 2-5 mg./day of DCA in the dog causes an increase in plasma volume after the first day, and a peak is reached after 3-7 days. Following this the increase is stabilized at about 5-10 ml./kg. above normal. The increased plasma volume is associated with marked retention of sodium and chloride, but these are rapidly eliminated with a return of all values to normal on cessation of treatment. If treatment with cortical extract is instituted after DCA injections are stopped, the plasma volume returns to normal. Some observations indicate that the changes in blood pressure cannot be directly correlated to the increased plasma volume (607).

The reduced blood pressure of the untreated adrenalectomized animal does not show spontaneous changes to any extent. That stimuli normally

evoking a pressure response in the intact animal are ineffective after adrenalectomy was established many years ago by Elliott (130,131). Hoskins (267) also noted a depression in irritability of the sympathetic nervous system in adrenal insufficiency. More recent studies by Cleghorn and collaborators have confirmed and extended these observations. Cats or dogs in adrenal insufficiency do not show a pressor response to stimulation of the splanchnic nerves; or to the intravenous injection of nicotine, posterior-pituitary extract, ephedrine, or barium. The pressure response to adrenaline, however, can be elicited. Stimulation of the carotid pressor reflexes by carotid occlusion caused a rise in blood pressure at a time when splanchnic stimulation was ineffective, but this disappeared with increasing adrenal insufficiency. Treatment with cortical extract brought about a return of the normal pressor responses (15,73,156,438,482). The failure of the various procedures to evoke a pressor response was not due to the exhaustion of sympathin (75,248) nor due to a failure of vascular constriction in the splanchnic area. Measurements have shown that splanchnic-nerve stimulation, Pitressin, and barium caused the normal constriction of the splanchnic vessels but did not cause a rise in arterial pressure (156). From these experiments it has been suggested that the absence of the pressor change is due to the inability of the heart to respond in the usual manner to increased peripheral resistance. Adrenaline, since it acts in part directly on the heart, does cause a pressor effect. Death in adrenal failure would therefore seem to be related to cardiac failure and a decreased capillary tone due to a breakdown of the cardioaccelerator and vasoconstrictor mechanisms.

### C. HYPERTENSION

The secretion of hormones which affect blood pressure by the adrenal medulla and cortex, and the low blood pressure associated with adrenal insufficiency suggested that the adrenals might be involved in the production of hypertension. Goldblatt (187) found that in dogs made hypertensive by renal-artery constriction, adrenalectomy was followed by a gradual reduction in blood pressure to normal values. Treatment with cortical extract was necessary to maintain a moderate degree of hypertension. On cessation of treatment the pressure would fall to subnormal levels. These results have been confirmed (32,85,161,441,694) and the effect is not dependent on removal of the adrenal medulla nor secondarily related to changes in gonad hormone production. Removal of only one adrenal does not affect existing hypertension. The effects of adrenalectomy are more pronounced than the fall in blood pressure produced by hypophysectomy. In the latter case the blood pressure may be restored by treatment with ACTH (13), and DCA also has a restorative action

(440b). Dogs first adrenalectomized show only a transitory and slight degree of hypertension following renal-artery constriction. The pressor response to renin is markedly reduced early after adrenalectomy, similar to the decreased response to Pitressin and barium. Large doses, however, will still evoke a response until the blood pressure falls to shock levels (162,462). In the animal maintained on DCA or cortical extract the normal pressor response to renin was found. The concentration of renin in the kidney of the cat was not found to be reduced following adrenalectomy (162). Hypertensive patients may show an exaggerated elevation of blood pressure following DCA injections (449a). It seems likely that the adrenal hormones probably do not participate directly in the production of hypertension but rather tend to maintain the animal in such a condition that it will respond in the normal manner to diminished renal blood flow.

#### D. CAPILLARIES

Related to the vascular failure of the adrenalectomized animal are associated changes in the blood. The loss of fluid from the blood and resultant increase in cells is reflected by the increased erythrocyte, hemoglobin, and hematocrit values. The reduced blood and plasma volumes cause an increased viscosity of the blood and hence a greater mechanical burden on the heart. The adrenalectomized animal apparently cannot compensate for this hemoconcentration and reduced blood flow, so that blood dilution does not take place unless suitable hormone replacement therapy is given (223,447). Many of these factors would cause a reduction in oxygen supply reaching the capillaries and might be expected to cause some functional impairment. Other evidence has been described which indicates that the capillaries may also be directly affected by the cortical hormones. The extensive observations of Swingle, Parkins, Taylor, and Hays on the vascular response of dogs in adrenal insufficiency have led them to believe that a breakdown of capillary function leads to death of the animal. The capillaries in such animals become atonic and dilated, causing a pooling of the blood with stasis. Anoxia is present and increased capillary permeability intensifies the hemoconcentration. The transfusion of hypertonic saline is followed by rapid improvement of the circulation, and a return to normal of the blood pressure and blood volume. Such an effect, however, is only transitory, since fluid again leaves the circulation and death follows. Cortin corrects such a loss of fluid (447,612). Any factor which tends to cause an additional burden on the already reduced capillary tone, such as certain shock procedures or the injection of intra-peritoneal glucose, causes a rapid deterioration in the animal's condition (447,612). Increased capillary permeability in adrenal insufficiency has been suggested by transfusion experiments (607). The normal dog or

adrenalectomized animal maintained by cortin showed no untoward effect of transfusion with dog serum. An animal in mild insufficiency with a normal appetite and a blood pressure of 75-80 mm. showed a marked reaction to a similar transfusion. Within a short time marked edema developed, especially in the eyes, lips, ears, legs, etc., and circulatory failure with a falling blood pressure to shock level took place in a few hours. The loss of the transfused serum in addition to the animal's own serum would indicate a marked decrease of capillary permeability. This could be corrected or prevented by cortical extract. The frequently noted increase in plasma protein of the adrenalectomized cat and dog has been shown to be due to the hemoconcentration (236). The plasma globulin fraction is therefore increased, but serum albumin, on the other hand, is lost despite the hemoconcentration. During cortical insufficiency, therefore, a marked depletion, up to 37%, of the serum albumin stores takes place, but not through the kidney. During treatment with DCA or cortin, hemodilution takes place with a lowering of the total plasma protein, and serum globulin. On the other hand, serum albumin concentration increases, indicating a direct effect of treatment on this fraction (236,349). Treatment of adrenalectomized rats with salt in paired-feeding experiments improved the plasma albumin concentration but did not restore it to normal (345). The protein content of lymph is also increased following adrenalectomy, and this is believed to be related to increased capillary permeability (87). Experiments involving the leakage of dyes through damaged capillaries of normal animals have suggested that cortical extract tends to prevent such changes. It is perhaps unfortunate that adrenalectomized animals were not included in the studies. The loss of trypan blue into a local area of lowered capillary permeability induced in the skin of a rabbit by the cutaneous injections of inflammatory exudate or leukotaxine was prevented by certain adrenal derivatives (159,405,406). Cortical extract was most effective but DCA, corticosterone, 11-dehydrocorticosterone, and Compound E were also active. Estrone, progesterone, and diethylstilbestrol were inactive. Similarly, the intracutaneous capillary response to peptone has been prevented by adrenal hormones (576). The loss of trypan blue into various areas of the body of the rabbit following intravenous injection has been found to be delayed or reduced by previous treatment with DCA, but not by testosterone or progesterone. The authors, however, do not believe the effect is one due to a reduction in capillary permeability (150). Cortical extract has also been found in the frog to prevent the hyperemia which can normally be induced by exposure, histamine, or local irritation (719). In the perfused leg of the frog it has also been noted that cortical extract effectively reduces edema formation, and such an action had been suggested as the basis for a possible method of assay (274).

## VII. Adrenal Cortex and Muscle Function

Since the original description of adrenal insufficiency by Addison, the associated debility, lack of muscular strength, and increased tendency to fatigue have been constant findings. In the untreated adrenalectomized animal, such symptoms are marked terminally, but even in the early stages of insufficiency it has been shown that spontaneous activity is decreased (125,297,489). Furthermore, muscle of the adrenalectomized animal has been found to fatigue more rapidly than does that of control animals after electrical stimulation. In 1927 Gans and Miley (168) found the muscle of the adrenalectomized rat able to perform only one-sixteenth the amount of work expected from control tests, and fatigue set in rapidly. In normal rats, however, cortical extract did not increase spontaneous activity (208). Normal dogs trained to run on a treadmill have been shown by Eagle, Britton, and Kline (126) to give an improved performance and greater energy output following cortical-extract therapy. Others have found similar results (17,275). Ettinger and Jeffs (135), however, found no improvement in exercise tolerance after prolonged intravenous infusion of cortical extract in dogs. Cortical extract had no effect on the fatigued isolated muscle of the frog (672). In man some reports have indicated an increase in muscular efficiency following cortical-extract treatment (37,231,263,411). Some cases of asthenia have also been benefited (192,271). A number of investigations have been reported on the influence of the adrenal cortex on neuromuscular transmission and on a comparison of the asthenia after adrenalectomy with the condition of myasthenia gravis (1d,154a,238a, 428a,540,670a).

The use of the rapid development of muscle fatigue after adrenalectomy has been adapted for assay purposes in a number of ways. In one case adrenalectomized rats have been made to swim to exhaustion in water (165). Comparison of swimming times before and after treatment were used as an index of the restorative action of the compound (673). Two tests using a direct measurement of muscle fatigue or work done have been developed and used extensively by two different groups of workers. The tests do not measure the same physiological response, since different types of compounds have different activities in the two tests. The test devised by Everse and de Fremery (142,143) has been used extensively for testing adrenal steroids obtained by Reichstein and collaborators. The adrenalectomized rat is used for this test 4 days after operation, and is prepared so that the tetanic contractions of the gastrocnemius of one leg are recorded following short, intermittent electrical stimulation by tetanizing current. Substances to be assayed are then injected once or twice daily for the following 4 days and another ergograph is obtained. The improvement in recovery of muscle fatigue is recorded. The minimum daily dose neces-

sary to cause effective recovery of fatigue so that a normal ergograph is obtained is referred to as one unit. In the Everse-de Fremery test, DCA is the most active steroid, so that a unit = 0.07 mg. The relative activity of some compounds is listed below (474,673):

Substance	Activity = 1 unit, mg.
DCA.....	0.07
Free desoxycorticosterone.....	Slightly less active than DCA
Amorphous fraction.....	Approx. 0.5
Corticosterone.....	0.8-1.00
17-Hydroxy,11-DCA.....	1.00
17-Hydroxycorticosterone.....	1.5-2.00
Compound E.....	2.00
Progesterone.....	Inactive

It may be noted that DCA and the amorphous fraction are the most active substances. The former is much the more active compound, and little difference is found in action of the other effective steroids. This would suggest the test is a reflection of the general condition and normal electrolyte balance of the animal, rather than an index of its carbohydrate metabolism.

A test for measuring the work capacity of rats was developed by Heron, Hales, and Ingle (257) and was used on adrenalectomized animals by Ingle in 1936 (277). Since then the test has been modified by Ingle on a number of occasions, until a final quantitative method was described in 1944 (293). This final procedure used the adrenalectomized, nephrectomized rat as a test animal, and the gastrocnemius muscle is made to contract by repeated faradic stimulation (3/sec.), against a 100-g. weight until exhausted. In an untreated animal exhaustion occurs in less than 10 hours, but with optimal treatment the muscle may continue to work for more than 24 hours. The test is started immediately after operation, in which case the substance to be tested is injected at the start of the stimulation and the injection dose repeated after 6 hours. Stimulation is continued until the muscle is exhausted, or for 24 hours, and the amount of work performed is recorded and calculated. Compound E has been used as a reference standard, and the work performance at a series of dose levels carefully assayed (293). In earlier reports the rats used for assay were adrenalectomized only and the fatigue test was done either immediately as described or after an interval of 7 days. In the latter case treatment was given daily over that period. In order to increase the stress to which the animal was subjected, and hence shorten the time of the experiment, injections of water were given during the test. Because of variables in the method all the reported assays

are not strictly comparable. However, the Table XV indicates the relative activities of some steroids taken from Ingle's reports (284,287,288,300). In one experiment the interesting observation was made that a mixture of Compound E and DCA was more effective than either substance when used alone (285).

In tests where treatment was given for 7 days following adrenalectomy, progesterone was found to have a slightly beneficial effect in a daily dose of 10.0 mg. The substance  $\Delta^4$ -pregnene-21-ol-3,20-dione acetate was active in a dose of 2-5 mg. daily.  $\Delta^6$ -Pregnene-3,21-diol-20-one acetate had a

TABLE XV

Substance	Dose (repeated after 6 hours) for same amount of work, mg.
17-Hydroxycorticosterone . . . . .	0.12
Compound E . . . . .	0.20
11-Dehydrocorticosterone . . . . .	0.25
Corticosterone . . . . .	0.50
DCA . . . . .	2.0-3.0 (only slight activ.)
DCA (sodium phosphate—aqueous or in oil)	2.0 (questionable activ.)
17-Hydroxy,11-DCA . . . . .	2.0 (questionable activ.)
Progesterone . . . . .	Inactive < 45.0
17( $\beta$ )-Hydroxyprogesterone	Inactive < 3.0
6-Hydroxyprogesterone	Inactive < 2.0
6-Hydroxy-11-desoxycorticosterone diacetate	Inactive < 2.0
$\Delta^4$ -17a-methyl-D-homoandrostene-17a-ol-3,17-dione . . . . .	Inactive < 10.0
Ethinyltestosterone . . . . .	Inactive < 20.0
Testosterone . . . . .	Inactive < 20.0
Testosterone propionate . . . . .	Inactive < 10.0
Estradiol benzoate . . . . .	Inactive < 10.0
Diethylstilbestrol . . . . .	Inactive < 50.0

slight action at 5 mg., but  $\Delta^6$ -pregnene-17,20,21 triol-3-one was inactive up to 5 mg. The first pregnene compound maintained body growth at a dose of approximately 0.25 mg. daily. The second was about half as active in this respect, while the last substance had no effect (283). Pretreatment with DCA was much less effective in sustaining work performance than Compound E (306). Delayed adsorption, therefore, was not the limiting factor in DCA administration, as suggested by Vögli (669,670).

The possible cause of the increasing muscular weakness after adrenalectomy has been investigated by various means. The similarity in symptoms between potassium poisoning and adrenal insufficiency suggested that potassium retention and electrolyte changes in muscle might be the eti-

ological factor in the cause of fatigue (728). Evidence, however, has been presented which indicates that muscle potassium falls during exercise in the untreated adrenalectomized animal (263a) and also that DCA therapy while effective in restoring normal electrolyte balance does not greatly improve work performance as measured by the Ingle technique. Treatment of the Addisonian, however, by salt or DCA does appear to reduce the symptoms of fatigue to a considerable degree, but this might be related to increased appetite and food intake. Pincus and Hoagland (459b-d) have reported experiments in which  $\Delta^5$ -pregnenolone therapy was believed to reduce psychomotor fatigue, although others do not agree on its action (192a), although differences in degrees of stress and motivation may well account for the discrepant results (459d). Ingle and collaborators have reported investigations designed to explain the mechanism underlying the development of muscular weakness after adrenalectomy. In direct measurements of the work performed in the rat it was found that treatment with sodium chloride or bicarbonate did improve the performance to a slight extent, but not to the same degree as did cortical extract (277,303). Cortical extract effectively improves the work capacity of the adrenalectomized (206,277,280) or adrenalectomized, nephrectomized rat (299,305) but the restoration does not reach the level of performance of a normal control animal. Similarly, adrenal steroids do not act as complete replacement therapy. Cortical extracts or adrenal steroids apparently do not improve the capacity of the normal animal to perform muscular work as judged by this test (293). The retention of potassium and increased level in the blood was not believed to be responsible for the decreased capacity for work of the adrenalectomized animal (303,305). Similarly, the lack of the adrenal medulla is not the causative factor, since following autogenous transplantation of the adrenals which causes degeneration of the medulla, the tolerance to fatigue is normal (296,297). After demedullation of the adrenals there is some decrease in the work capacity and adrenaline has a slight restorative effect, but only of a transitory nature.

The relation of the depletion of carbohydrate stores and hypoglycemia to the inability to perform prolonged work, however, seems well established. As early as 1926 Swingle (602) commented on the finding of low blood sugar associated with the onset of muscular weakness. Following the performance of work there is a marked fall in blood sugar in the untreated adrenalectomized or adrenalectomized, nephrectomized rat. Values of 37 milligrams per cent may be obtained and may be compared to values of 73-87 milligrams per cent for control animals (293,305). Furthermore, infusion of hypertonic glucose will restore the work capacity of the adrenalectomized animal to practically normal (293,303). It will be noted that the activity of the various adrenal steroids in restoring work capacity,

as described, is proportional to their ability to build up carbohydrate and to maintain blood sugar values, as will be discussed later. The activity of four adrenal steroids and two extracts measured by the muscle work test has been compared to their ability to cause glycogen deposition in the adrenalectomized rat. In all cases a parallelism in activity in the two tests was observed (440a). Associated with the onset of fatigue and hypoglycemia in the untreated animal is a profound circulatory collapse, and it has been suggested that failure of the cardiovascular mechanisms may be another factor in the induction of fatigue (206,293,303). It is of interest to note that the reduced work capacity of the hypophysectomized rat has been shown to be greatly improved following pretreatment with purified ACTH (302).

### VIII. Adrenal Cortex and Carbohydrate Metabolism

That the adrenal glands exerted an effect on carbohydrate metabolism was noted in 1908 when Bierry and Malloizel (31) reported that after adrenalectomy in dogs the blood sugar might fall to one-half or one-fifth of its original level. Shortly after, Porges (462) confirmed this observation and Schwartz (539) noted the disappearance of glycogen from the liver of the rat following adrenal removal. Kuriyama (342) later reviewed the earlier work and showed that adrenalectomized rats would maintain liver glycogen provided they took food normally. After fasting, however, difficulty in storing glycogen was noted. Some years later the reduction in blood sugar of the adrenalectomized dog and cat again received comment, but it was believed to be predominantly a terminal change (239,502), or, exaggerated by the trauma of the operation (19). In 1926 Zwemer (721) showed that the oral administration of glucose to adrenalectomized cats would prolong their survival to some 220 hours, about four times longer than untreated controls. A careful study of adrenalectomized rats was made about this time by Cori and Cori (94). They showed that, after fasting, the liver glycogen of the adrenalectomized rat disappeared, and a low blood sugar (53 to 79%) was present. Muscle glycogen, on the other hand, was unaffected. Such animals could form glycogen when they were fed glucose. Britton and Silvette and colleagues have studied carbohydrate changes after adrenalectomy, and the effects of treatment with cortical extracts in many species. The liver glycogen was consistently found to be depleted and some degree of hypoglycemia was present in adrenalectomized rats, guinea pigs, marmots, cats, dogs, and monkeys (42,43,45,46,48,49). Animals with low liver glycogen did not show the usual hyperglycemia following emotion or adrenaline injections (46,49). Cortical extract was found to cause an increase in blood sugar and glycogen deposition in normal as well as adrenalectomized animals (43,44,49), whereas treatment with salt had no effect.

on blood sugar (49). Monkeys apparently showed the most severe effects after adrenal removal, the blood sugar falling to as low as 18 milligrams per cent. In some cases the animals were continuing to take food but this did not prevent symptoms attributed to hypoglycemia. As a result of these studies it was suggested that the cause of death after adrenalectomy was predominantly a failure of carbohydrate metabolism.

TABLE XVI  
CARBOHYDRATE LEVELS OF NORMAL AND ADRENALECTOMIZED RATS AND MICE<sup>a</sup>

Fasting, hr.	Glycogen, mg. %		Blood glucose, mg. %
	Liver	Muscle	
Mice			
0	2840	435	
0	<i>2177</i>	<i>479</i>	
6	171	332	
6	<i>86</i>	<i>238</i>	
12	252	298	
12	<i>102</i>	<i>269</i>	
24	346	228	
24	<i>44</i>	<i>158</i>	
Rats			
0	1780	590	124
0	<i>2310</i>	<i>533</i>	97
24	25	520	72
24	<i>16</i>	<i>432</i>	54
48	233	507	80
48	<i>70</i>	<i>358</i>	30

<sup>a</sup> Values for adrenalectomized animals are given in italics.

Other studies have tended to clarify the effects of adrenalectomy, and it is now apparent that there is a certain degree of species difference, and the time of onset of hypoglycemia is directly related to the duration of fasting. In the cat the terminal stages of adrenal insufficiency are associated with hypoglycemia, and this can be corrected by cortical extract (57,725,726). In the dog the changes in blood sugar are only slight unless there is either associated trauma, as in a one-stage operation or inanition (19,222,444,502).

In the rat changes are more marked, even though the animal's general condition may be maintained by salt (56,376).

In 1940 Long, Katzin, and Fry (367) presented a very detailed review and study on the influence of the adrenal cortex on carbohydrate and protein metabolism. From the stimulus given by these workers interest was revived in this problem, which has been widely studied in the past few years. Long and collaborators have confirmed the findings that in the adrenalectomized rat and mouse the blood sugar and storage of glycogen are essentially normal provided the food consumption is kept normal by maintaining the animals on sodium chloride. After fasting, however, the

TABLE XVII  
EFFECT OF CORTICAL EXTRACT AND ADRENAL STEROIDS ON GLYCOGEN

Notes and treatment	Glycogen, mg. %	
	Liver	Muscle
Normal mice		
24-hr. fasted controls . . . . .	346	288
Adrenaline (1:1 million), 0.25 ml./hr., 8 times . . . . .	315	
Cortical extract, 0.25 ml./hr., 10-12 times	2986	223
Corticosterone, 0.025-0.5 mg., 8-10 times	1890	
Dehydrocorticosterone, 0.25-0.5 mg., 8 times . . . . .	2260	
Fed controls .. . . . .	2840	435
Cortical extract, 0.25 ml./hr., 8-24 times	9196	1014
Adrenalectomized mice		
24 Hr. fasted controls . . . . .	44	158
Cortical extract, 0.25 ml./hr., 12 times . . . . .	2370	182

liver glycogen falls dramatically and is associated with hypoglycemia. Muscle glycogen also decreases significantly. The typical changes are shown in the abbreviated tables constructed from their paper (Tables XVI and XVII). The effects of cortical extract and certain adrenal steroids may be seen in Table XVII. The mice were fasted for 12 hours before the start of injections and until killed at 24 hours. Similar effects of adrenalectomy on glycogen in rats were noted by Evans (138). That corticosterone possessed an action on carbohydrate metabolism was noted in early experiments with this compound (475).

Abelin has recently published some interesting observations on the action of dietary carbohydrate in initiating a pituitary-adrenal mechanism con-

cerned with the storage of glycogen. He has shown that following the feeding of glucose, sucrose, etc. to fasted rats there is a fall in adrenal cholesterol associated with the increase in liver glycogen (1). Such treatment also augments the reduction in adrenal cholesterol which occurs in response to ACTH (1b). The rise in blood sugar from the ingestion of carbohydrate may therefore stimulate ACTH with a subsequent release of cortical steroids, which in turn facilitate the building of liver glycogen (1a).

Extensive observations on carbohydrate metabolism in cases of Addison's disease have been made by Thorn, Koepf, Lewis, and Olsen (651). The changes found include a low fasting blood glucose level, readily induced severe hypoglycemia and the development of symptoms at a lowered threshold, altered glucose tolerance curve, and decreased hyperglycemic response to adrenaline. Effective treatment with cortical extract reversed these findings. The most active therapeutic agents were cortical extract, Compound E, or corticosterone.

#### A. METHOD OF ASSAY: LIVER GLYCOGEN

The response of the liver glycogen of the adrenalectomized animal has formed the basis of an assay method which on careful study has proved to be of great value and precision. Besides being used to compare the effect of the various adrenal steroids, it has been widely adopted for assaying extracts prepared from the urine for their cortical activity.

Reinecke and Kendall (476) first described an assay method using the adrenalectomized rat. On the fourth day after operation the animal was starved and injections were given hourly; the animal was killed 1-2 hours after the last injection for estimation of liver glycogen. With cortical extract it was found that doubling the amounts of extract used produced a difference in the liver glycogen deposited of approximately four times. Olson and collaborators (439) modified the above test by feeding the rats a high protein diet after operation and under their conditions a strictly linear relationship existed between the logarithm of the total dose of extract and the amount of liver glycogen. Because the adrenalectomized mouse may be some ten times more sensitive than the rat, a number of workers have used this species. A detailed discussion of this method of assay by four groups of workers (Venning and Kazmin, Dobriner, Lieberman, and Eggleston; Grisivold and Forbes, and Dorfman, Shipley, Schiller, and Ross) has been recorded (86). Recently Venning, Kazmin, and Bell (664) have found that the administration of glucose following adrenalectomy greatly increases the sensitivity of the test, so that 0.1 ml. cortical extract caused a measurable amount of glycogen deposition. Similarly, the activity of an extract of 500 ml. urine could be estimated by this method. Compound E is usually used as a standard for the expression of results. The level of

liver glycogen is raised from 4-10 mg./100 g. body weight in untreated control mice, to approximately 100 mg./100 g. body weight following treatment with 0.050 mg. Compound E (86). Dorfman, Ross, and Shipley (112) have also worked out the details of the glycogen deposition assay method in adrenalectomized mice and noted the extent of the variations in response. They have found the relationship between the response and the logarithm of the dose to be linear and the slopes similar for pig adrenocortical extract, 11-dehydrocorticosterone, and Compound E. Eggleston, Johnston, and Dobriner (127) have in addition used a titration method for assay of liver glycogen deposition in adrenalectomized mice. In this the dosage of steroid is adjusted until an amount is found which maintains a liver glycogen of from 70 to 130 mg./100 g. body weight. Both methods are described in detail, and dose-response curves for Compound E recorded.

#### B. COMPARATIVE ACTIVITY OF ADRENAL STEROIDS ON LIVER GLYCOGEN

Using the adrenalectomized rat for assay, it has been found that 17-hydroxycorticosterone, Compound E, 11-dehydrocorticosterone, and corticosterone were the most active compounds, in the order listed, although the greatest difference was slight, probably less than 50% by weight. 17-Hydroxycorticosterone, however, exhibited a regression line having a slope over 1.5 times and a potency 1.49 times that of corticosterone. DCA, allopregnane-3,11,17,20,21-pentol, 17-hydroxy-11-dehydro-3,4,5-tetrahydro-3( $\beta$ )-hydroxycorticosterone (Compound G), and 11-dehydro-3,4,5-tetrahydro-3( $\beta$ )-hydroxycorticosterone (Compound H) were inactive (440,477). Augmentation of potency occurred when corticosterone and 17-hydroxycorticosterone were administered simultaneously (440). Such an effect had been predicted because of the relatively high potency of cortical extract (477). Recently Pabst, Sheppard, and Kuizenga (440a) found that 17-hydroxycorticosterone was three times and Compound E twice as active as 11-dehydrocorticosterone or corticosterone when assayed on a large series of adrenalectomized rats. An extract of hog adrenal was approximately twice as active as one of beef adrenal—96 g. original hog tissue and 216 g. beef tissue being equivalent to 1 mg. Compound E.

In the mouse assay rather different comparative effects have been obtained. Thus it has been found that Compound E was rather more than three times as active as 11-dehydrocorticosterone, 0.040 mg. of the former being more effective than 0.10 mg. of the latter. A dose of 0.4 ml. cortical extract was about as active as 0.40 mg. Compound E (86,664). In another report (112) approximately the same relationship was noted between the potency of Compound E and 11-dehydrocorticosterone. A pig adrenocortical extract was highly potent. Doses as low as 0.01 ml. caused a greater glycogen deposition than 10 or 20  $\mu$ g. Compound E. One ml. of such a

cortical extract has been found to be as effective as from 0.776 to 0.995 mg. Compound E (114).

In humans with Addison's disease Compound E (33 mg.), was more active than corticosterone (80 mg.), in affecting carbohydrate metabolism. Cortical extract (50 ml.) was the most potent, whereas DCA (30 mg.) was ineffective (651). Progesterone has been shown in large doses of 50 mg. to increase the liver glycogen of normal ferrets but not of rats (180).

This effect on liver glycogen is not, however, confined to adrenal steroids. The natural estrogens, or diethylstilbestrol have similar actions. This effect may, however, be an indirect one through the pituitary and adrenals, since the action does not take place in adrenalectomized or hypophysectomized animals. Details of these observations are given in the review by Swingle and Remington (620).

### C. CARBOHYDRATE UTILIZATION

Whereas the experimental results just outlined clearly demonstrate an important role of the adrenal cortex in carbohydrate metabolism, more detailed studies have been made on the mechanism involved in these changes. Thus the low blood sugar and depleted glycogen content of the liver of the fasted adrenalectomized animal appear definitely related to the increased oxidation of glucose. Although the adrenalectomized animal apparently utilizes glucose at an increased rate, if sufficient carbohydrate is given, glycogen deposition will take place. Animals maintained on salt and with a normal food intake therefore show essentially normal carbohydrate levels. The intestinal absorption of glucose and carbohydrate after adrenalectomy may be altered so that the rate of absorption is reduced as insufficiency increases (6,52,94,270,398). If the animal is maintained on sodium salts the rate is normal (6,9,71,109,519). The theory advanced by Verzar (667) that the adrenal hormones are essential in the phosphorylation of glucose in the intestine has not had general acceptance (620).

Even the fasted adrenalectomized animal shows deposition of glycogen if given sufficient glucose (9,18,94,109,138,367,460), so that glycogen synthesis can occur in the absence of adrenal hormones. Some observations indicate that the rate of glycogen deposition may be abnormally low after adrenalectomy (5,1138,354) and conversely Corey and Britton (92) found in a cat liver preparation perfused *in vitro* with glucose - gum - Ringer solution that glycogen formation was increased to 50 to 100% within 15 minutes when cortical extract was added to the perfusing solution. DCA, on the other hand, had no effect.

At the same time it seems well established that more than normal amounts of glucose are being oxidized so that for glycogen to be deposited larger amounts of glucose are required after adrenalectomy. In balance

studies on adrenalectomized rats Evans found 51 mg./100 g. body weight of injected glucose unaccounted for when compared to controls; such a difference was ascribed to increased oxidation of glucose (138). Evans (137) in the previous experiments on phlorizin diabetes had found, as had others (264), that adrenalectomy in the rat caused a reduction in sugar output. Wells (680) found similar changes after adrenal removal and showed that corticosterone and Compound E restored the glucose excretion to normal levels. DCA in large doses was only partially effective, and the amorphous fraction in comparative dosage to corticosterone, when standardized on the adrenalectomized dog, had little effect. Lewis, Kuhlman, Delbue, Koepf, and Thorn (354) also studied the excretion of administered glucose to phlorizinized adrenalectomized rats, and found that such a preparation utilized 43% of the glucose compared to 19% in control animals. Treatment with cortical extract, but not with DCA, corrected this alteration. In experiments with dogs after phlorizin poisoning, the blood sugar of the adrenalectomized animal reached a hypoglycemic level within 20 hours, whereas in intact animals the time was approximately doubled. The adrenalectomized dog shows typical symptoms of hypoglycemia at a time when the blood sugar was higher than that necessary to give comparable symptoms in normal dogs. Apparently the threshold for hypoglycemic symptoms is reduced by adrenal removal. A similar finding was noted for rats, and treatment by cortical extract or Compound E, but not DCA, was effective preventative therapy. In support of the theory of increased oxidation after adrenalectomy are some observations on the respiratory quotient (R.Q.). These studies indicate that in adrenal insufficiency there is a tendency for the R.Q. to rise and that cortical extract or corticosterone cause a lowering of the R.Q. (322,354,367,519,651). Russell (520), however, from a consideration of her own work and that of others, believes that the adrenalectomized animal when in the best of condition does not have an increased rate of carbohydrate utilization. This view is supported by reports of a normal R.Q. for the adrenalectomized animal (51,137,519,650), and the finding of practically normal deposition of glycogen or recovery of absorbed carbohydrate in glucose-fed adrenalectomized rats (8,109,519).

### *1. Glycogenolysis and Cortical Extract*

The increased blood sugar and liver glycogen noted after treatment with cortical hormones are apparently associated with an increased rate of glycogen formation, a decreased tendency for liver glycogen to break down to glucose, and a marked reduction in glucose oxidation by the body, as well as with increased conversion of protein to carbohydrate, to be discussed later. Seckel (549) found that liver slices taken from adrenalect-

tomized rats showed a reduced rate of glycogenolysis when cortical extract was added. This result has also been obtained when a perfused rat liver preparation was tested (92). The decreased oxidation of glucose following cortical hormone administration has been indicated in experiments previously mentioned, in studies on the R.Q. changes, and on animals after phlorizin treatment. A more direct experiment has been cited by Ingle (286,294). Normal rats were force-fed a diet from which 15 g. glucose could be derived daily. This amount was at least 50% less glucose than such animals could normally handle without showing glycosuria. If such animals received 5 mg. daily of either 17-hydroxycorticosterone or Compound E they developed severe glycosuria, excreting up to 12 g. glucose daily. Such glycosuria could not be controlled by 100 units of protamine-zinc insulin daily. Changes in the urinary nitrogen output indicated that not more than 1 g. of the urinary glucose could have been formed from protein. It was suggested, therefore, that these steroids inhibited the utilization of glucose. Similarly, cortical extract and 11-oxygenated adrenal steroids increase the glucose excretion in partially depancreatized or adrenalectomized-depancreatized rats, and such a change cannot wholly be explained by the conversion of protein to carbohydrate (282,306a,309,367). The diabetes induced by adrenal steroids has been compared to that following pancreatectomy in the rat (307).

## *2. Insulin and Cortical Hormones*

The relationship of the pancreas and adrenal cortex to carbohydrate metabolism has been studied intensively since the original observations in 1934 by Long and Lukens (368,369,371) and Hartman and Brownell (227). A review of the early work is contained in one of these papers (371). The demonstration that adrenalectomy attenuated the severe diabetes of the totally depancreatized cat or dog has been confirmed by other workers and for other species such as the rat and toad (262,269,367,372,375). Similarly, adrenalectomy reduces diabetes produced in the rat following alloxan treatment (316). The reduction in food intake, however, which follows adrenalectomy of the rat made diabetic by alloxan, is apparently an important consideration, since it was shown by Janes, Dawson, and Myers (315) to be mainly responsible for the reduction in blood and urinary glucose rather than the effect of adrenalectomy *per se*. However, in forced-feeding, constant-food-intake experiments, Ingle and collaborators have found that an amelioration of pancreatic diabetes in rats occurs following adrenalectomy (306a,309). The mild diabetes of the adrenalectomized-depancreatized animal can be exaggerated by the injection of cortical extract in cats or dogs (375) or more readily in rats (367). Diabetes of the partially depancreatized rat has been shown by Long, Katzin, and Fry

(367) and Ingle (282,309) to be increased in severity by corticosterone, 11-dehydrocorticosterone, but not by desoxycorticosterone or progesterone. The same effects have been obtained in other species (110). Similarly, in the adrenalectomized-depancreatized rat, corticosterone, 11-dehydrocorticosterone, and Compound E increased the diabetic condition, whereas progesterone was non-active (309,367). A number of studies have been made on the action of insulin and cortical hormones in rats from which the liver, pancreas, and viscera have been removed (304a,306b,495a,519). The results indicate that adrenalectomy causes an increased rate of glucose utilization. Infused glucose disappeared more rapidly provided excessive amounts were not injected and the simultaneous administration of insulin did not alter this effect (304a). Cortical extract and insulin when given together caused a rise in blood glucose above that of control animals (306b). The interpretation of these results is complicated by the fact that the operative procedure causes considerable shock to the animal and the effects of cortical extract may be related to the alleviation of shock symptoms (495a,520).

### *3. Insulin Sensitivity*

The adrenalectomized animal is much more sensitive than the normal to insulin injections. Part of this increased sensitivity is due to removal of the adrenal medulla. Swann and Fitzgerald (601) have reviewed some of the early reports and have added experiments in which adrenals were transplanted so that no medullary tissue would survive. They found that normal fasted rats convulsed following an average dose of 2.2 units of insulin/kg. Adrenalectomized rats maintained in good health by a suitable diet were 24 times more sensitive, a dose of only 0.09 units of insulin/kg. being required to cause convulsions. In animals with transplanted adrenals for the first 7 weeks the dose was 0.32 units/kg., but this subsequently increased to a constant level of 1.0 units/kg. The adrenalectomized dog is similarly extremely sensitive to the hypoglycemic action of insulin, 0.25 units/kg. being followed by severe convulsions (222,354). Prevention of the signs of insulin hypoglycemia may be accomplished by pretreatment with 70 ml. cortical extract or 12 mg. Compound E, but DCA in large doses has no protective action (354).

The prevention of the hypoglycemic action of insulin by adrenal steroids in normal animals has been used as a method of assay. Jensen and Grattan (317) found that suitable doses of cortical extract or corticosterone, but not DCA, would effectively prevent insulin convulsions in mice. The ACTH of the anterior pituitary was also effective. In further studies it was shown that the convulsive effect of 2 units of insulin/kg. in male adult mice was inhibited by a total dose of 0.5 mg. Compound E, 17-hydroxy-

corticosterone, corticosterone, and its acetate. On the other hand, in 2.0-mg. doses, desoxycorticosterone, DCA, progesterone, and a number of estrogens and androgens were inactive. The active steroids were also found to increase the liver glycogen of fasted mice and this seemed related to their anti-insulin potency. The anti-insulin effect of pituitary ACTH was believed to be through liberation of active adrenal steroids (193,194).

As previously noted, Ingle (294) was able to induce a temporary diabetic condition in normal rats on a high-carbohydrate diet by large doses of certain 11-oxygenated steroids. In this case the action of intrinsic insulin was apparently inhibited. It was also shown that, if the diabetes was controlled by large doses of insulin—up to and more than 100 units, an increased dosage of the adrenal steroid was followed by a further increase in sugar excretion in the urine. Apparently the administered insulin and adrenal steroid could be balanced to neutralize their individual opposing actions on carbohydrate metabolism.

The actual mechanism of the anti-insulin action of adrenal steroids has been clarified by the fundamental observations recently published by the Coris and associates. In the initial paper, Price, Cori, and Colowick (464) showed that insulin and anterior-pituitary extract act antagonistically on the hexokinase reaction. This substance catalyzes the reaction glucose + adenosine triphosphate → glucose-6-phosphate + adenosine diphosphate. This reaction represents the first step in the utilization of glucose by the tissues, its transformation to glycogen, and its oxidation. This reaction was found to be inhibited by anterior-pituitary extract, either *in vitro* or *in vivo*, but the inhibition could be counteracted by insulin. Tissue extracts prepared from rats made diabetic by alloxan showed an absence of hexokinase activity, but not if the animal was receiving insulin injections prior to the experiment. Price, Slein, Colowick, and Cori (465) have continued the study using adrenal hormones. It was found that cortical extract greatly intensified the inhibiting effect of added or previously injected anterior-pituitary extract on the hexokinase reaction, although it alone did not affect hexokinase in normal extracts. Whenever inhibition by cortical extract was observed, insulin invariably released the inhibition, about 50 µg. insulin being required to neutralize 0.1 ml. of Upjohn's cortical extract. Curiously enough, the amorphous fraction possessed the activity of the cortical extract and corticosterone, 11-dehydrocorticosterone, or Compound E were without effect. These preliminary observations, demonstrating inhibition of the hexokinase reaction by anterior-pituitary extract (which in turn is intensified by an adrenal principle) and the opposing neutralizing of the inhibition by insulin, are of fundamental importance for a complete understanding of the hormonal control of carbohydrate metabolism. These findings have been expanded and described in greater detail

in more recent papers by Colowick, Cori, and Stein (85a) and Krahl and Cori (337a).

#### *4. Anterior Pituitary - Adrenal Relationship*

The preceding observations make it increasingly clear that, in any consideration of the adrenal cortex on carbohydrate metabolism, the hormones of the pituitary gland must also be considered. Although a discussion of the pituitary is outside the scope of this chapter, a few pertinent observations may be cited. Detailed reviews have been published by Houssay (268), Russell (518), Thomson (629), Haist (205), Long, Katzin, and Fry (367), Young (715), and Swann (599).

Removal of the anterior lobe of the pituitary gland causes marked changes in carbohydrate metabolism, which in many respects are similar to those following adrenalectomy. The hypophysectomized animal has a low blood sugar and a brief period of fasting is followed by depletion of liver glycogen and hypoglycemia. The R.Q. is high and direct experiments indicate that glucose is being oxidized more rapidly than normal. The sensitivity to insulin is markedly increased following hypophysectomy, and treatment with anterior lobe extracts reduces such increased sensitivity to normal. Certain pituitary extracts contain a glycotrophic substance which antagonizes the action of injected insulin even though they may not affect the fasting blood sugar *per se*. Some evidence indicates this effect to be through an adrenal mechanism. The effect of purified pituitary hormones on muscle and liver glycogen have been reported by Herring and Evans (259). ACTH on prolonged administration maintained glycogen levels during fasting. Bennett and Li (24) found that ACTH treatment increased the urinary glucose and nitrogen in alloxan-diabetic rats. The increase in nitrogen was not sufficient to indicate that the increased glucose was derived from protein. Removal of the pituitary gland from animals made diabetic by a previous pancreatectomy, as originally shown by the classic observations of Houssay and Biasotti, causes an alleviation of the severity of the diabetic condition. Fasting of the Houssay animal also results in hypoglycemia, and this may be prevented by adrenocortical extract. Pituitary ACTH causes a similar effect, and it may induce a marked increase in the severity of the glycosuria and the diabetic condition. It would appear, therefore, that many of the effects of hypophysectomy on carbohydrate metabolism may be due indirectly to the subsequent adrenal hypofunction. However, a considerable amount of evidence exists that pituitary factors other than ACTH have an effect on carbohydrate metabolism. It has been shown that anterior-pituitary extract may cause glycosuria in the partially depancreatized, adrenalectomized animal so that an adrenal action could be ruled out. Furthermore, the dramatic property of crude pituitary ex-

tracts to inhibit the production of insulin by a direct action on the pancreas and to produce a permanent diabetic condition has been extensively studied by Young and collaborators. The observation that anterior-pituitary extracts which are diabetogenic in the Houssay preparation were inactive in the adrenalectomized, depancreatized animal unless small amounts of cortical extract were given simultaneously is of interest in view of the Coris' observations.

### 5. Muscle Glycogen

The effect of adrenal or pituitary preparations on muscle glycogen does not necessarily parallel the effect on this substance in the liver. The adrenal appears to be chiefly concerned with liver glycogen, whereas the anterior pituitary affects muscle glycogen, although a synergistic action also takes place. Muscle glycogen is only slightly reduced in the adrenalectomized fasted animal until marked exhaustion of the carbohydrate stores has taken place. Animals which have not been maintained in good condition before the start of the experiment show greater alterations in muscle glycogen, especially after fasting. It seems clear, however, that adrenalectomized animals maintained on a proper diet have normal levels of glycogen in both liver and muscle. Treatment with cortical extract will maintain the level of muscle glycogen following adrenalectomy, but has little effect on muscle glycogen in hypophysectomized animals. It may cause an increase in normal mice (367). Liver glycogen, on the other hand, is increased in the normal, adrenalectomized, or hypophysectomized animal by cortical extract. The anterior pituitary appears to exert a much greater effect on muscle glycogen than does the adrenal cortex, and has been discussed by Russell (519). In the normal animal fed glucose, anterior-pituitary extract caused an increased deposition of muscle glycogen, whereas cortical extract increased liver glycogen. Both actions apparently were related to a depression of the utilization of carbohydrate. Pituitary ACTH also increased the deposition of muscle glycogen. In adrenalectomized animals, however, anterior-pituitary extract did not affect muscle glycogen unless synergized by small doses of cortical extract given simultaneously. In fasted rats, anterior-pituitary extract did not affect liver glycogen but maintained muscle glycogen even in the absence of the adrenal cortex or in the adrenalectomized, hypophysectomized rat (521). In the latter preparation, purified ACTH had no effect on muscle glycogen (25).

## IX. Adrenal Cortex and Protein Metabolism

The observations of Long, Katzin, and Fry previously referred to (367) focused attention on the role played by the adrenal cortex in protein metabolism. From complete metabolic data determined on fasted animals

treated with cortical extract, it was clear that the increased carbohydrate stores could not be due to translocation of carbohydrate but were related to the formation of new carbohydrate from protein. When fasted, normal, adrenalectomized, or hypophysectomized rats were treated with cortical extract the typical increase in liver glycogen and over-all body carbohydrate was found. At the same time the excretion of urinary nitrogen was increased, indicating an increased breakdown of protein. The glucose-nitrogen ratios obtained in the first two groups of animals showed that 53 to 66% of the extra protein catabolized was converted into glucose. Since the R.Q. did not increase there was no indication of a marked increase in carbohydrate oxidation (322,367). In fasted adrenalectomized dogs treated with corticosterone (15 mg.) or cortical extract (50 ml.), an increase in nitrogen excretion has been noted at the same time as a reduction in the R.Q. from the level of untreated controls (354). Sprague (590) has also shown an increased excretion of urinary nitrogen after cortical-extract treatment in fasting adrenalectomized rats. Rats fed a high-carbohydrate diet also show an increased urinary-nitrogen output following treatment with corticosterone or 17-hydroxycorticosterone (308).

In the case of the fed animal Wells and Kendall (682) have shown that after phlorizin treatment the adrenalectomized rat fed casein excreted the expected amounts of glucose and protein similar to the intact animal. It was found that the metabolism of exogenous protein was not dependent on the adrenocortical hormones. When the supply of exogenous protein was limited, however, and endogenous protein must be mobilized, the failure of the adrenalectomized animal to form glucose from protein became apparent. Ingle and collaborators (305a,306a,310) also have found that the force-fed, saline-treated, adrenalectomized rat is able to catabolize protein in a normal manner and shows a normal urinary excretion of nitrogen. Stoerk, John, and Eisen (598a) have fed labeled glycine to rats and found that the rates of synthesis and breakdown of serum proteins were not affected by adrenalectomy or treatment with cortical extract.

The untreated adrenalectomized animal might be expected to show the opposite effect to that following cortical-extract treatment, a reduction in urinary-nitrogen output. Reports on this, however, appear conflicting. Long and associates (367,370) have found a decreased urinary-nitrogen excretion in adrenalectomized animals. Rubin and Krick (516) noted a negative nitrogen balance in adrenalectomized rats which was reduced by salt therapy. A relationship between the decreased nitrogen output and reduced food intake was noted. Food consumption of the adrenalectomized rat, although greatly improved by treatment with saline, has been found by most workers not to be restored to normal (345,516,712), although contrary results have been reported (196). Sandberg and Perla (528), however,

found an increased excretion of urinary total nitrogen in the rat following adrenalectomy. Urea, creatinine, and sulfate excretion increased, whereas ammonia and uric acid were unchanged. Noble and Toby (436a) have recently reported that nitrogen excretion in the saline-treated adrenalectomized rat was somewhat increased by the fifth post-operative day but returned to normal values in 4 weeks. Nitrogen retention was not observed except for a few hours following operation. Evans (137) found that after

TABLE XVIII  
EXCRETION OF GLUCOSE AND NITROGEN IN PHLORIZINIZED RATS

Condition and daily dose	Glucose excreted, mg./100 g. body weight	Nitrogen excreted, mg./100 g. body weight
Normal controls . . . .	621	182
	574	162
Demedullated controls . . .	624	172
Adrenalectomized . . . .	142	46
Saline . . . .	313	92
Compound E, 2 mg. . . .	641	191
Compound E, 1 mg. . . .	473	135
Corticosterone, 2 mg . . .	598	166
11-Dehydrocorticosterone, 2 mg . . . .	583	165
DCA, 2 mg. . . .	410	120
DCA, 1 mg.. . . .	375	112
DCA, 2 mg. . . .	263	83
Amorphous, 3 dog units . . . .	237	63
DCA, 1 mg., plus compound E, 1 mg.....	572	172
Thyroidectomized . . . . .	477	139
Thyroidectomized, adrenalectomized		
Saline . . . . .	140	61
Compound E, 2 mg. . . . .	382	103
Compound E, 2 mg. plus thyroxine, 0.1 mg.	721	190
Hypophysectomized . . . .	148	57
Compound E, 3 mg. . . . .	412	170
Compound E, 2 mg. plus thyrotrophic...	625	196

phlorizin treatment, adrenalectomized rats showed a markedly reduced glucose and nitrogen excretion. The glucose-nitrogen ratio was lowered by adrenalectomy. This observation has been confirmed and extended in that Compound E or adrenocortical extract, but not DCA, were found to restore to normal the glucose and nitrogen excretion in such a preparation and cause a significant rise in the glucose-nitrogen ratio (354). Wells (680) has also reported similar results. Wells and Kendall (682) have reported the effect of many adrenal steroids on adrenalectomized, phlorizin-

ized fasted rats, and their various results have been combined in Table XVIII. The urine collection was made after phlorizin administration on the second day of fasting, but therapy was given on the first and second days. These observations show clearly the effect of certain adrenal steroids in causing an increased excretion of glucose and nitrogen in a relatively constant ratio. It may also be noted that Compound E and DCA given together have a potentiating action. Compound E was relatively inactive in the adrenalectomized, thyroidectomized rat unless thyroxine was given simultaneously. It was effective, however, in the hypophysectomized animal.

#### A. NITROGEN EXCRETION AFTER TRAUMA

Extensive observations on protein metabolism after trauma have been made on animals and humans since Cuthbertson (97a,b) emphasized the marked negative nitrogen balance which is consistently noted (2a,50a, 55a,83a,270a,310,436a,686a). Since trauma also induces hypertrophy of the adrenal cortex (559), breakdown of lymphoid structures (689a), depletion of ascorbic acid and cholesterol in the adrenal cortex (531c), and the excretion of a substance in the urine having biological activity similar to 11-oxygenated adrenal steroids (572a,663a), there is considerable evidence to suggest that adrenal stimulation takes place. Most workers have considered the possibility that the negative nitrogen balance after trauma is due to stimulation of adrenocortical hormones. Thus the urinary nitrogen might be increased due to the dissolution of lymphocytes and lymphoid tissues. This source of the urinary nitrogen, however, would appear relatively small when one considers the large and protracted loss of nitrogen which may occur in certain clinical conditions. There appears to be no direct evidence that an increased production of 11-oxygenated adrenal steroids acting on tissue protein causes increased catabolism and resulting negative nitrogen balance after trauma. Although the injection of these steroids into animals may cause increased nitrogen excretion, Noble and Toby (436a) have shown that increased protein catabolism after trauma does take place in the adrenalectomized rat. In these experiments the increased metabolism of protein was indicated by an accumulation of end products in the blood, since the depressed kidney function of the adrenalectomized animal did not allow urinary excretion to take place. Treatment with doses of cortical extract insufficient to affect protein catabolism *per se* restored kidney function with a resulting excretion of nitrogen, after trauma, of the same magnitude as that found in intact controls. Ingle, Ward, and Kuizenga (310), similarly have reported that an increased excretion of nitrogen after leg fracture in the adrenalectomized rat takes place if the animal is treated with a basal level dose of cortical extract.

### B. DEPANCREATIZED ANIMALS

Animals made diabetic by partial pancreatectomy, fasted, and treated with cortical extract showed a marked increase in glucose and nitrogen excretion in the urine, again indicating an increased catabolism of protein (309,367). Houssay rats or cats treated with cortical extract showed similar results (367,391). Conversely, the excessive conversion of body protein to glucose which takes place after pancreatectomy is greatly reduced by a subsequent adrenalectomy. As a result, after fasting the blood sugar falls to a low level and the excretion of nitrogen in the urine is diminished (371).

### C. PITUITARY HORMONES AND NITROGEN EXCRETION

Nitrogen excretion may also be influenced by pituitary hormones. As would be anticipated, active adrenocorticotropic extracts should cause an increased excretion of nitrogen through their adrenal action. Although this aspect has not received much attention experimentally due to the lack of highly purified pituitary hormones, it has been shown that severe weight loss follows treatment with ACTH (141,415,433) as it does after certain 11-oxygenated adrenal steroids are given. The excretion of uric acid was markedly increased in normal persons but only slightly in Addisonian patients following injections of ACTH. Creatinine excretion tended to remain constant or fall. The uric acid-creatinine ratio in such cases was of greater diagnostic value than alterations in total urinary nitrogen (154e).

Crude anterior-pituitary extracts and purified growth hormone are apparently antagonized by ACTH. When the two antagonistic hormones are given together weight increase in the hypophysectomized animal is reduced or abolished (403,433). Crude pituitary extract induces in fed animals a retention of nitrogen, and in some species hyperglycemia and glycosuria, but in fasted normal or adrenalectomized rats hypoglycemia, acetonuria, and a reduction of urinary nitrogen is found (110,166,210,347, 413,415,519,626). The retention of nitrogen is believed to be due to the growth hormone, as other highly purified pituitary fractions were shown by Marx, Magy, Simpson, and Evans (402) to be only slightly active or inert. Purified growth hormone causes marked nitrogen retention (191a).

### D. MECHANISM OF ACTION

The mechanism by which the adrenal cortex acts on protein metabolism is not yet established, although some suggestive observations have been made. It would seem possible that the adrenalectomized animal cannot deaminate body amino acids at a normal rate, and that the deaminated acids are only slowly converted to glucose. In each case it would seem that the rate of the reaction is primarily affected. Furthermore, the difference

in the ability of the animal to handle exogenous and endogenous protein suggests that in the fasting animal an interference with some stage of the breakdown of the protein molecule to amino acids may occur in the absence of the cortical hormones. The evidence for these possibilities follows.

### 1. *Deamination of Amino Acids*

A failure of the liver or kidney of the adrenalectomized animal to affect deamination could explain the decreased excretion of ammonia and urea characteristic of insufficiency. Conversely, cortical extract by increasing deamination would result in increased production of urea and glucose. Samuels, Butts, Schott, and Ball (526) found that fasted adrenalectomized rats fed alanine did not form as much liver glycogen as did intact controls, although some glycogen deposition did take place. Evans (139), on the other hand, from studies on the blood amino acid and urea levels of nephrectomized, adrenalectomized rats, concluded that injected **DL**-alanine disappeared at the same rate, and the urea formed was the same as in control animals. Lewis, Kuhlman, Delbue, Koepf, and Thorn (354), in more extensive experiments on phlorizinized rats, showed that following alanine treatment the adrenalectomized animal did not show the expected increased excretion of glucose and nitrogen. The excretion of glucose was less than that occurring when equivalent amounts of glucose were administered under comparable conditions, so that increased oxidation was not an explanatory factor. The failure of deamination was corrected by cortical-extract therapy. Wells and Kendall (682), however, have found that the nonfasted adrenalectomized rat can utilize ingested casein in a normal manner.

Experiments with tissue slices have also indicated a failure of deamination in the absence of the cortical hormones. Jimenez-Diaz (318) found a reduced rate of deamination in kidney slices from adrenalectomized cats. Russell and Wilhelm (522,523) in extensive experiments on adrenalectomized rat kidney slices have found that oxygen uptake in the absence of substrate or in the presence of the substrates **DL**-alanine, **L**(+)-glutamic, or  $\alpha$ -ketoglutaric acid (the product of deamination of glutamic acid) is significantly less than normal. As judged from ammonia or glucose formation, the rates of deamination were less than normal. These reduced activities were restored to normal or above normal by previous treatment of the adrenalectomized animals by cortical extract or DCA. Liver slices from adrenalectomized rats apparently behave in a different manner, since it has been reported that **DL**-alanine and **D**-glutamic acid in the substrates do not form carbohydrate more rapidly following previous treatment of the donor animal by cortical extract, although under similar conditions pyruvate did so (335). The failure of the kidney tissue to cause deamination

and the restoration to normal by DCA is of interest, since this steroid does not cause increased protein breakdown in metabolism studies. It is probable that the results obtained on isolated tissues may only reflect some of the many changes which occur in the intact animal.

## 2. Conversion of Deaminized Acids to Glucose

Considerable evidence has been reported which indicates that after adrenalectomy there is a reduced rate of conversion of keto and hydroxy acids to glucose. Buell, Anderson, and Strauss (56), using fasted adrenalectomized rats, found that lactic acid was not converted to liver glycogen at a normal rate. They suggested and reviewed older reports which indicate that in adrenal insufficiency there is a partial failure of lactic acid production and a limited resynthesis of phosphocreatine. Apparently the human in adrenal insufficiency also cannot form glucose from lactic acid (651). Lewis *et al.* (354), using the phlorizinized adrenalectomized rat, also noted that glucose formation from lactate and pyruvate was abnormally low, but treatment with cortical extract or Compound E restored the conversion to normal, although DCA was ineffective. The possibility of reduced absorption in these experiments has been cited in criticism by Russell and Wilhelmi (522), since they found that kidney slices from adrenalectomized rats could form carbohydrate normally from pyruvate and succinate. Liver slices from adrenalectomized animals, however, do not form carbohydrate from pyruvate and lactate at a normal rate. Pretreatment with cortical extract has been found to increase the rate of formation of carbohydrate in both normal or adrenalectomized rats (335).

The above reports indicating a reduced ability of the adrenalectomized animal or its tissues to deaminate amino acids and convert deaminized acids to glucose are difficult to interpret in view of the observations by Wells and Kendall (682) and Ingle *et al.* (306a) that the fed adrenalectomized rat can convert protein to glucose in a normal fashion. In such a case the absorbed amino acids from the gastrointestinal tract were apparently handled in a normal manner, and there was no evidence of reduced glucose formation as might have been expected. Although the fasted adrenalectomized animal may not break down body protein to amino acids at a normal rate, it seems curious that such amino acids when formed should not be handled as readily as those obtained from exogenous sources. At the present time there seems to be no simple theory of adrenal action on protein metabolism which will explain all the experimental data which has been presented. The suggestions that the 11-oxygenated steroids or "S" hormones may be concerned with an antianabolic action rather than a catabolic one on protein, as suggested by Albright (2a), or may have both actions (55a,686a) have been used to explain some of the actions of

injected adrenal steroids and the changes in nitrogen balance occurring after trauma. Although these theories are of interest they do not satisfactorily explain all the experimental results outlined. White and Dougherty (689b) have shown that in the mouse the thyroid as well as the adrenal has an influence on the mobilization of nitrogen after fasting. The adrenalectomized animal drew on the carcass and liver but not on its lymphoid tissue for its nitrogen demands, whereas the intact mouse used the latter source as well. After thyroidectomy nitrogen was obtained only from the liver and lymphoid tissue and if adrenalectomy was performed in addition only liver nitrogen was affected. The thyroid therefore appeared to control the catabolism of protein in muscle tissue during fasting and there was some evidence that thyroid activity was influenced by adrenocortical secretion. Adrenalectomy has also been shown to prevent the deposition of protein in the liver remnant after partial hepatectomy. Treatment with cortical extract but not DCA restored deposition to normal (25a).

## X. Adrenal Cortex and Enzymes

A number of reports have been summarized which concern some of the enzyme systems which may be affected by adrenalectomy or cortical hormones. In rat kidney the concentrations of flavin-adenine-dinucleotide (512) and cozymase (517) are unaltered after adrenalectomy. The respiratory metabolism of various tissues appears to be depressed after adrenalectomy. The  $QO_2$  of testes, brain, kidney, and liver has been reported as depressed or in some cases unaltered (96,261,522,652). Tipton (653,653a) has shown that the activity of cytochrome oxidase and the concentrations of cytochrome c in heart, kidney, and liver were decreased in adrenalectomized rats. Treatment with sodium chloride partially prevented the decrease, but adrenocortical extract was almost completely effective in preventing the observed changes. Folley and Greenbaum have confirmed that a decrease in liver arginase follows adrenalectomy (154b,c). Liver arginase levels of adrenalectomized rats were not elevated by cortical-extract therapy although the lowered kidney arginase was somewhat restored (334b). Vail and Kochakian (662a) have reported that liver alkaline phosphatase is slightly increased and kidney alkaline phosphatase somewhat reduced in the rat following adrenalectomy. Cortical extract caused a marked increase in activity of the enzyme in liver but DCA was inactive. The decreased level in the kidney could be prevented by salt or DCA therapy and cortical extract did not cause increased levels. Acid phosphatase was unaltered by any of the above procedures. Other workers have previously reported a more marked decrease in kidney alkaline phosphatase following adrenalectomy in the rat, guinea pig, and cat (156a,318,342a). Serum peptidase in mice has been shown to be increased following a single injection of cortical extract or ACTH (264a).

## XI. Adrenal Cortex and Fat Metabolism

The influence exerted by the adrenal cortex on liver fat and the production of ketone bodies is not so fully understood as the effect on carbohydrate and protein metabolism. However, there is considerable evidence that the adrenals are a factor in fat metabolism. The subject has been reviewed by Ingle (290).

### A. EFFECT ON LIVER FAT

The effect of adrenalectomy on certain methods of increasing liver fat have been studied by a number of workers. Certain anterior-pituitary extracts have marked properties to produce ketosis in fasting animals (14,23a,b,28,60). Ketone body production by such extracts does not take place after removal of the liver (412) and is reduced following adrenalectomy. MacKay and Barnes (386) also reported that the liver of adrenalectomized rats contains less fat and is practically unaffected by anterior-pituitary extract. Fry (164) found that the fatty infiltration of the liver of the fasted rat produced by certain anterior-pituitary fractions did not occur in previously adrenalectomized animals. Whereas the normal livers showed fatty acids of 6.9 grams per cent and a total fat of 9.0 grams per cent, livers from the adrenalectomized rats assayed at 2 to 4 grams per cent, a figure comparable to the values for untreated controls. Removal of the adrenal medulla was not responsible for such an altered response.

The increase in liver fat which occurs in the remainder of the organ after partial hepatectomy in fasted rats has been shown to be greatly reduced by adrenalectomy or the administration of glucose, but not by choline (388). Adrenalectomy has also been stated to reduce the fat deposition in the liver after phosphorus poisoning (666). Samuels and Conant (527), from experiments in which normal and adrenalectomized rats were fed high-fat diets and the response of liver fat and acetone body excretion to fasting was measured, suggest that in the absence of adrenocortical hormones there is a defect in the transport of fat from the depots to the liver.

### B. EFFECT ON KETOSIS

The ketosis which normally follows the injection of certain substances or altered physiological state of the animal has been shown to be markedly reduced by adrenalectomy. Thus the increased urinary ketone body output following fasting of the fat-fed or normal female rat was reduced by adrenal removal (385). The ketonuria following fasting of the pregnant rat was similarly reduced (383). The injection of anterior-pituitary extract did not produce the expected control levels of urinary ketone bodies in adrenalectomized rats (163,164,385). The ketonuria of the phlorizinized rat was found to be reduced by adrenalectomy (683), as was that found in rats exposed to low oxygen tension. Removal of the adrenal

medulla alone was not responsible for this effect (137). The ketonurias of the depancreatized cat and dog are also abolished or reduced by subsequent removal of the adrenals (371,372).

The experiments quoted on the excretion of ketone bodies after adrenalectomy may be questioned following the observations of MacKay and Barnes (385) that in the adrenalectomized rat some degree of ketonemia may be present following anterior-pituitary extract injections, although ketonuria is absent. Mirsky (414) extended and confirmed these observations and concluded that adrenalectomy increased the renal threshold for ketone bodies. Further confirmation has been reported (389,428,574). In later experiments it was shown, however, that adrenalectomy definitely reduces the rate of acetone body utilization (422). Shipley (571) could not demonstrate any alteration in renal threshold for acetone bodies in the adrenalectomized rat, but found the ketonemic response to anterior-pituitary extract reduced but not abolished. From measurements of arteriovenous differences it has been found that the adrenalectomized fasting rat showed an increase in the rate of acetone body utilization (390). Broda (50) also found a lower level of ketones in the adrenal venous blood when compared with the arterial blood of the dog.

### C. CORTICAL HORMONES AND KETOSIS

Judging from the effect of adrenalectomy one would expect cortical-extract injection to induce ketosis. The evidence for this, however, is not clear-cut. MacKay and Barnes (384) reported that cortical extract induced an increased ketonuria in fasting female rats, but the report has been criticized because the extract contained ketone substances (571). Grollman (198) and Shipley and Fry (573) were unable to confirm the ketogenic action of cortical extracts, and the latter similarly could not induce a significant ketonuria by corticosterone (3 mg.), Compound E (2.6 mg.), or DCA (10 mg.). Ingle and Thorn (309) found an increase in excretion of ketone bodies in some partially depancreatized rats, and in adrenalectomized, partially depancreatized rats, but this was associated with increase in glucose and nitrogen excretion and loss in weight of the animals. Using phlorizinized animals Wells, however (683), has found that Compound E (2 mg.), corticosterone acetate (2 mg.), or DCA (6 mg.) markedly increased the excretion of ketone bodies in fasting adrenalectomized rats. The increased glucose excretion associated with the DCA treatment was relatively slight. Similar results have been obtained by other workers for the rat, and also in an adrenalectomized, phlorizinized dog (354).

Kendall (86) has reported interesting changes induced in the mouse following prolonged treatment with pellets of adrenal steroids. From analyses of the whole animal it was found that, in certain strains of mice,

treatment, especially with corticosterone or 11-dehydrocorticosterone, caused a marked increase in the percentage of fat and reduction in protein of the carcass. It was suggested that the increased formation of carbohydrate over periods of 4 to 7 weeks led to increased conversion to fat. Mice treated in this way with corticosterone or Compound E, but not 11-dehydrocorticosterone, showed a high incidence of degenerative and necrotic areas of skeletal and cardiac muscle.

#### D. PHOSPHORYLATION

Verzar and others (310a,409,410,666,668,669) postulated a theory that the adrenal cortex was essential for the phosphorylation of carbohydrate or fatty acids in the intestinal mucosa. Verzar believed that the retarded rate of absorption of fat in the adrenalectomized animal could be corrected by cortical extract or flavin and phosphoric acid. Most workers have been unable to substantiate these observations and Verzar's conclusions appear to be unfounded. Details of these observations have been reviewed (598,620).

### XII. Adrenal Cortex and Resistance

Ever since the effects of adrenalectomy were first studied in animals it has been obvious that the operation induced a greatly reduced resistance to many forms of stress. The operation itself was frequently rapidly fatal, depending upon the amount of operative trauma. The deleterious effect of postoperative infection and changes in temperature have been noted. At the present time an extensive list can be made of hormones, drugs, poisons, toxins, infections, altered environmental conditions, and different types of trauma, which are all fatal to adrenalectomized animals, yet scarcely interfere with the well-being of a normal one. As a generalization, it may be asserted that any substance will cause mortality in the adrenalectomized animal in a dose of one-fifth to one-twentieth that required to kill the normal. It is not necessary that such substances belong to those generally classified as toxins, since many normally occurring hormones and organic and inorganic substances are effective. Although the removal of the adrenal medulla at first was thought to be responsible for the increased susceptibility of the adrenalectomized animal, it now seems clear that this is of minor importance. Treatment with cortical extract will restore the resistance following adrenalectomy, although similar treatment will not as a rule raise the resistance of an intact animal above normal. Various methods of assay have been advocated, based on the alterations in resistance. The following discussion will be limited to various substances and procedures which have most commonly been tested on adrenalectomized animals.

### A. RESISTANCE TO VARIOUS AGENTS

#### 1. *Drugs, Poisons, and Pharmacological Agents*

Early observations on the sensitivity of adrenalectomized rats to various agents are complicated by the fact that since the operation had a low mortality the animals presumably had some residual functioning cortical tissue. Nevertheless, these animals were hypersensitive. Boinet in 1896 (34) first demonstrated such increased sensitivity; and a review of the earlier experiments of this type may be found in a paper by Torino and Lewis (352,656). Some of the various agents which have been administered to adrenalectomized animals are: adrenaline, atropine, cobra venom, cardiac toxins, digitoxin, curare, papaverine, codeine, veratrine, potassium cyanide, nicotine, and acetonitrile. In all cases increased sensitivity after adrenal extirpation was noted. In the case of the convulsants, strychnine, picrotoxin, and tetanus toxin, there was no increased sensitivity. Morphine has been studied by a number of workers, and extensively by Lewis. The lethal dose for the adrenalectomized rat is approximately one-tenth that for the normal animal (543,656).

Possibly of greater physiological interest are the studies on the toxicity of histamine. Dale in 1920 (99) showed that in adrenalectomized cats the hypotensive effect of histamine was ten times greater than in normal animals, and this was soon confirmed (327). Banting and Gairns (19) found the adrenalectomized dog also highly sensitive to the toxic action of the same drug. In the rat adrenalectomy reduces the fatal dose of histamine by some ten to twenty times (97,396,545) although considerable controversy exists over the role played by the adrenal medulla (705,710). Ingle (278), in a recent paper, found that whereas rats without the adrenal medulla were less sensitive to histamine than were totally adrenalectomized animals they were still more sensitive than intact controls. Hypophysectomized rats are also more sensitive to injected histamine, and protection is afforded by adrenocorticotropic extracts (432,450,452,453). Cortical extract similarly raises the lowered resistance of the adrenalectomized or hypophysectomized animal (451,455). It has been suggested that the protection afforded adrenalectomized rats against histamine by cortical extract be used as a method of assay (455,545). Rose and Browne (510) found that the distribution of injected histamine in the tissues was unchanged following adrenalectomy, but the rate of disappearance was markedly retarded, especially in the kidney. In further studies it was found that in the adrenalectomized rat there is a marked increase in the histamine content of the gastrointestinal tract, liver, and lungs (400,511). Following treatment with either cortical extract or DCA the ability to inactivate histamine is restored to normal. The dose required, however,

was considerably greater than that needed for maintenance of growth in the adrenalectomized animal (509). Blood histamine levels have been found to be raised after adrenalectomy (696). In one report (454) normal rats and mice were found to tolerate larger doses of histamine after treatment with saline, saline and DCA, or saline and cortical extract.

As previously pointed out, such a normal body constituent as urea is much more toxic when given to adrenalectomized rats. Of the hormones, estrogens appear to be very poorly tolerated after adrenal removal (559).

## 2. Bacterial Toxins and Foreign Proteins

The adrenalectomized animal has also been shown by numerous workers to have an increased susceptibility to the lethal effect of various bacterial toxins. Diphtheria toxin has been shown to kill adrenalectomized rats in doses which do not affect intact animals (255,352). Furthermore, in such cases the toxin remains in the blood due to defective elimination or destruction (23). Cortical extracts have been stated to increase the resistance of normal animals (723) but other reports have not confirmed this action in guinea pigs for diphtheria, tetanus, botulinus, or pneumococcal toxins (547,690). The adrenalectomized rat has similarly been shown to be more susceptible to killed *Staphylococcus aureus* and streptococci (544); cortical extract restored resistance to normal (242). Adrenalectomy has been stated to make the rat more susceptible to infection with tubercle bacilli (597), and cortical extract has been thought to confer some protection to the same infection in guinea pigs (463). Typhoid vaccine has also been found to be poorly tolerated by adrenalectomized animals (313, 314) although high titers of antibodies can be induced over a period of time (314). Cortical extract effectively restores the resistance to normal (134, 241,242,546), but DCA is ineffective (134). Recently an assay method involving the protection of adrenalectomized rats against typhoid vaccine has been reported by Lewis and Page (353). The activity of cortical extracts paralleled their carbohydrate activity, but this action was not responsible for the protection afforded. In some cases of active infectious processes, cortical extract has been thought to be beneficial (685,686,691).

Adrenalectomized rats have been found to show an increased sensitivity to anaphylactic shock from horse serum, although the loss of the adrenal medulla may have some part in the altered sensitivity (154,707). Conversely, cortical extract has afforded some protection against the anaphylactic reaction of egg albumin in normal guinea pigs (704) and against anaphylaxis in dogs (124). Ingle (292) has found that large doses of cortical extract, especially that prepared from hog adrenals, gave increased protection against peptone shock in normal rats, whereas DCA was ineffective.

### 3. Environmental Temperature

The deleterious effect of temperature changes on the survival of adrenalectomized animals has been noted since the earliest detailed observations were reported (131). In 1926 Belding and Wyman (23) noted the marked fall in rectal temperature which followed exposure to a temperature of 3°C. in adrenalectomized rats. This was confirmed by Wyman and tum Suden (708), who further noted that the effect was due to removal of the adrenal cortex rather than the medulla. Hartman, Brownell, and Crosby (229) later showed that, whereas the fall in rectal temperature in normal rats exposed to cold was only 1°C., adrenalectomized animals maintained on saline showed a drop of 12°C. and many died after similar treatment. Treatment with cortical extract of adrenalectomized animals prevented most of the drop in temperature (to 3°C.), but adrenaline was not effective. The low temperatures recorded were believed to be due to failure of heat production. The general metabolism was reduced 10-20% in operated animals, but restored by cortical-extract treatment. Hartman (225) also showed that cortical extract prevented mortality from cold in adrenal-deficient rats, and suggested the response be used as an assay method. Subsequent publications (228) have confirmed these observations, and tend to show that the failure of the adrenalectomized animal to rapidly increase heat production when exposed to cold leads to a rapid fall in body temperature and death. The associated general muscular asthenia and circulatory deficiency are contributing factors. Young rats are more affected than old, and the effect of removal of only one adrenal definitely reduces heat production from exposure to cold, although the rectal temperature may remain normal (265,266,495,679). The hypophysectomized rat is similarly very sensitive to cold, and adrenocorticotropic extracts have been shown to give protection in these rats, but have no protective action on adrenalectomized animals. Cortical extract, however, protected both preparations (660). Heat production in adrenalectomized pigeons maintained on salt has been found to show little change from normal (493).

Selye and Schenker (569) outlined an assay method using the response of young adrenalectomized rats exposed to a temperature of 2-5°C. Treatment at the start and during the test with cortical extract afforded protection, whereas control untreated animals died after 6-10 hours exposure. By this method a protective action for as little as 0.03 ml. cortical extract could be demonstrated. Roos (507) has suggested modifications for increasing the accuracy of the test. Kendall (329) has reported that corticosterone (16 µg.), Compound E (20 µg.), and the amorphous fraction (13 µg.) afforded protection to cold. Zarrow (717) noted that DCA in a total dose of 1-2 mg. or progesterone (2 mg.) were active in the cold test using adrenalectomized mice, but only if the animals were pretreated be-

fore exposure. Recently, Dorfman, Shipley, Schiller, and Horwitt (114) have published extensive observations on the cold test as a method of assay. They have concluded that rats are preferable to mice for the test, but considerable variation in response occurs from group to group. To obtain an accurate assay required large numbers of animals. A standard curve for 11-dehydrocorticosterone was used for comparative assays on urinary cortin extracts. The same group of workers (113) have compared the cold test and glycogen deposition test in adrenalectomized mice with the following results. The equivalent amounts of the various steroids were estimated, using 1  $\mu\text{g}$ . 11-dehydrocorticosterone as a standard for the cold test. In the cold test Compound E was the most active, requiring approximately 0.3  $\mu\text{g}$ . or one-third the dose of 11-dehydrocorticosterone. Corticosterone required 3.8  $\mu\text{g}$ . and DCA approximately 4.3  $\mu\text{g}$ . In terms of Compound E, which was the most active, 11-dehydrocorticosterone, corticosterone, and DCA were one-third, one-twelfth, and one-thirteenth as effective, respectively. In the liver glycogen assay, 11-dehydrocorticosterone was one-fourth as active as Compound E.

The adrenalectomized rat apparently survives poorly at a high temperature (37°C.), as well as at low temperatures (679). Protection against high temperatures is afforded to some extent by sodium chloride, but to a greater degree by DCA. Hemoconcentration occurs most markedly in the untreated animals (256). Sodium retention appears to be the most important factor in protection against heat in contrast to cold where the carbohydrate-effective steroids are most beneficial. Cortical extract was not found to increase the performance or subjective feelings of men working in an atmosphere of moist heat (417) and DCA therapy has been shown to reduce by 30% the salt lost in sweating (343a).

#### 4. *Reduced Oxygen Tension*

The reaction of the normal and adrenalectomized animal to reduced pressure is of special interest, since this form of stress seems to be markedly influenced by and have a direct effect on the adrenals. Exposure of an animal to low oxygen tension is followed by pathological changes and enlargement of the adrenal glands, as well as thymus atrophy (16,355,624, 649). The tolerance of the adrenalectomized animal is extremely low. Adaptation to repeated exposures occurs readily in normal, but not in adrenalectomized, animals unless they are treated with cortical extract (624,649). This general picture is that described by Selye and termed the "alarm reaction" (559); it will be described later.

Evans (136) first reported the interesting observation that following an exposure for 24 hours to reduced oxygen tension fasted rats showed marked increase in blood sugar and liver and muscle glycogen. On the

other hand, adrenalectomized animals similarly exposed showed no change in carbohydrate metabolism. In further studies he found such changes in the normal rat associated with an increase in excretion of nitrogen and ketone bodies and a raised R.Q. The hypophysectomized animal behaved like the adrenalectomized. From these results it appeared that liberation of adrenal steroids were increasing protein breakdown and increasing the formation of carbohydrate (137). Thorn, Jones, Lewis, Mitchell, and Koepf (649) have extended these studies and have confirmed the effects of short exposure to low oxygen tension. Rats, rabbits, monkeys, but not dogs, show the same increase in liver glycogen, and blood glucose and increased excretion of nitrogen. The increase in glycogen apparently varies inversely to the oxygen tension. After repeated exposures to low oxygen tension, however, the blood sugar is found to be low and liver glycogen is reduced below normal values. In another report, however, rats which had been acclimatized did not develop secondary hypoglycemia after acute anoxia (663). Adrenalectomized animals were unable to stand comparable exposures or adapt to low pressure unless treated with cortical extract. DCA was not effective. Changes in electrolytes also occurred after exposure and the normal animal showed a marked increase in the excretion of sodium, chloride, and potassium. In adrenalectomized animals only potassium excretion was increased and cortical extract treatment restored the alteration to normal (355).

Although cortical extract will restore the lowered susceptibility of the adrenalectomized animal to reduced oxygen tension, initial experiments to attempt to increase the resistance of intact rats (137) or to improve work performance at reduced pressures were unsuccessful (115). Recently, however, positive results have been obtained by Thorn, Clinton, Davis, and Lewis (633). In these experiments the mortality at different oxygen tensions was determined on normal rats. Whereas treatment with DCA or Compound E (0.5 mg.) did not improve the animals' tolerance, cortical extract in oil reduced the mortality by 50% or more. These results may have a more general significance, since attempts to increase the resistance of normal animals to varied forms of stress by cortical extract have uniformly been negative, but in no case has the extract been given in oil. The altered conditions for absorption may be an important factor in increasing the effectiveness of this type of therapy. The resistance of normal rats to anoxia was found by Li and Herring (356) to be increased following treatment with purified ACTH.

##### 5. *Trauma*

The general picture of adrenal insufficiency and the terminal profound collapse led many of the early workers to comment on the resemblance

of the condition to secondary shock. Swingle, Pfiffner, Vars, Bott, and Parkins (617) have listed many changes which are comparable in the two conditions. The observations that adrenalectomized animals were highly susceptible to traumatic and other procedures causing shock led to the belief that the condition might respond to treatment by cortical extract. With the added impetus to the study of shock due to the war, many workers contributed observations concerning participation of the adrenal glands in shock. Although many reports are conflicting, it seems that certain cortical hormones will restore to normal the lowered susceptibility to shock of the adrenalectomized animal, but they will not increase the normal resistance of the intact animal. In some forms of shock encountered clinically, adrenal hormones have possibly brought about improvement, but their effect has not been dramatic. The possibility exists that insufficient dosage has been used, or that some of the less readily available steroids would be effective in shock.

Many experiments clearly show the increased susceptibility of the adrenalectomized animal to trauma. This change occurs rapidly after adrenal removal. Rich (488) found that adrenalectomized cats immediately following the operation withstood intestinal manipulation as well as intact animals. 17 hours following adrenal removal, however, the animals were definitely more susceptible to trauma. Swingle and Parkins (606) compared normal and adrenal-insufficient dogs. Amounts of trauma applied to intestine, muscle, or testes which scarcely affected normal animals were found to cause shock and death in those adrenalectomized. Further extensive studies from this laboratory showed that adrenalectomized dogs were much more susceptible than normal ones to hemorrhage. They would tolerate only approximately one-eighth of the blood removal and such hemorrhage was not followed by blood dilution and restoration of blood volume as seen in normal animals. Cortical-extract therapy prevented or restored to normal the altered response (618). DCA when administered to adrenalectomized dogs previously maintained on cortical extract also afforded protection against hemorrhage (483). Shock following trauma in adrenalectomized dogs was not found to be due to aggravation of the existing alterations in sodium, chloride, or potassium, or due to hemoconcentration *per se*. Treatment with hypertonic saline gave only temporary improvement, in contrast to cortical extract. Circulatory failure in adrenalectomized dogs following trauma only occurred when there was associated local fluid loss. Effective therapy appeared to depend on the improvement of the circulation, increase in blood volume, and restoration of capillary control (447,483,613). Shock following large doses of adrenalin in the adrenalectomized dog could also be effectively treated by cortical extract (446). In further studies it was found that DCA exerted a

protective action against the circulatory collapse in the adrenalectomized dog induced by intraperitoneal glucose injection, adrenaline, or muscle trauma, but was not effective against intestinal trauma. With the last form of trauma, however, corticosterone treatment was effective (604). Following hemorrhage, the inability of the adrenalectomized animal to sustain prolonged vasoconstriction or to show blood dilution was corrected by either DCA or cortical extract (445). The high mortality following a single-stage bilateral adrenalectomy in the dog could not be reduced by DCA but was reduced by cortical extract. On the other hand, if a local nerve block was performed at the same time DCA was then an effective form of therapy. Cortical extract, therefore, and not DCA, appeared to have the ability to reduce the lethal effects of injury to nerve structures (445). It is of interest to note that DCA has been shown to afford some protection to the lethal effect of x-radiation of mice (129b).

The adrenalectomized rat is similarly more susceptible to trauma. Freed (157) found the reduced tolerance to muscle trauma to be improved by saline therapy. The increased sensitivity to trauma is marked 3 days after adrenalectomy, but is slight immediately after operation (678). The severity of the shock following muscle trauma was believed to be proportional to the rate of fluid loss into the damaged tissue. Removal of one adrenal also reduced the resistance to trauma (251). In acute exsanguination experiments, adrenalectomized rats, even when maintained on salt, cannot be bled to the same extent as those treated with DCA or intact controls (250). A quantitative method of applying trauma to the rat in a rotating drum, with minimal degrees of hemorrhage and without anesthesia, has been described by Noble and Collip (435). Adrenalectomized rats tolerated only about half the amount of trauma as did normal animals. Pretreatment with cortical extract restored the resistance to normal levels, whereas DCA caused only slight improvement. When normal rats were treated in the same manner they tolerated only very little more trauma than controls. Active corticotrophic extracts of the anterior pituitary were no more effective than cortical extract in normal animals (436). Clarke and Cleghorn (72) have considered the biochemical changes found in adrenal insufficiency and in traumatic shock in rats and dogs. Although the same changes in the blood and tissues were not found in the two conditions, the deleterious effect and significance of the increased blood potassium was discussed, especially in its relationship to visceral congestion. Root and Mann (508) made observations on the behavior of the capillaries after intestinal manipulation or following the release of tourniquets. Cortical extract had no corrective action on the capillary dilatation, congestion, and stasis.

Other experiments assessing the value of cortical extract or DCA ther-

apy on shock in intact animals have yielded conflicting results. Despite some enthusiastic reports, the over-all evidence indicates that such therapy is of little value. The many publications have been critically analyzed by Huizenga, Broffman, and Wiggers (273). A cortical extract and plasma mixture has thus been reported of value in preventing shock produced by the injection of toxic products produced in closed intestinal loops (260), and saline and cortical extract was thought to lessen the toxemia following high intestinal obstruction in dogs (703). DCA has been shown to partially prevent the fall in plasma volume following distention of the bowel in dogs, although mortality was not reduced (151). Cortical extract has also been shown to prevent the drop in plasma volume occurring during ether anesthesia (376a,469). DCA has been reported to be of value in restoring to normal the blood sodium level of cats after scalding (488). The circulatory collapse and shocklike prostration which follow regional venous occlusion in dogs has been stated to be favorably affected by adrenal hormones. DCA therapy preceding and after such a procedure apparently reduced mortality, but did not affect the local fluid loss (36,321, 575). These beneficial effects, however, have not been confirmed in duplicate experiments (605). Shock following limb ischemia was not influenced by treatment with cortical extract, Compound E, DCA, or corticosterone (291,605,620). Selye and Dosne (562) and Selye, Dosne, Bassett, and Whittaker (563) believed cortical extract and corticosterone to be effective against the toxic effects of formalin injection or intestinal crushing, but DCA was inactive. Weil, Rose, and Browne (677) presented evidence to show that rabbits treated with DCA and cortical extract were resistant to intestinal trauma, whereas DCA treatment alone was of no benefit. Ingle and Kuizenga (301) could not detect any beneficial effect of treatment with hog adrenal extracts or DCA on survival following burn shock in intact rats, in confirmation of other work (466). Shock following hemorrhage in dogs seems definitely not to be influenced by pre-treatment or therapy with cortical extract (253,272,273,605).

The clinical use of cortical extract in shock or related conditions is unsettled, although evidence is against this as a useful form of therapy (273). In severe burns there is perhaps some indication that serum electrolyte changes may be reduced or prevented, and the efficacy of transfusion enhanced (485,486,697). The inconsistency of the results may be seen by reference to the following papers (51,311,323,324,336,437,454,468,469,472, 485,548,697-699).

#### B. ROLE OF ADRENALS IN DAMAGE AND RESISTANCE

The general lowering of the resistance of the adrenalectomized animal to all forms of harmful treatment, as described, has led to considerable

speculation on the role of the adrenal in the normal response to damaging stimuli. The effectiveness of cortical extract in restoring the reduced resistance after adrenalectomy is well established, but the fact that treatment of normal animals does not as a rule improve their resistance makes it difficult to assess what function the adrenals may assume in the response of intact animals to damage. Selye and collaborators have published extensively on the response of the normal animal to noxious agents. During the adaptation of the animal to such stress they have observed three successive stages. The first, of brief duration, is the "alarm reaction," during which all harmful agents induce changes which are believed to represent a mechanism of increasing general resistance. This phase is characterized by changes usually described as occurring in secondary shock, and is followed by the "counter-shock phase." At this time, some 48 hours later, the signs are reversed and adrenal enlargement with histological evidence of increased activity, involution of the thymus and lymphatic organs, leucopenia followed by leucocytosis, and increase in blood volume, blood sugar, and chlorides occur. Continued treatment leads to the "stage of resistance," during which the morphologic lesions regress and resistance to the specific agent, but not other agents, is pronounced. Finally, the resistance is lost and a "stage of exhaustion" develops, with death of the animal. This thesis embraces the suggestion that the countershock phase is due to adrenal stimulation and increased production of hormones, and finally with exhaustion a condition comparable to adrenal insufficiency ensues. Details of these observations are contained in the recent review by Selye (559).

The large number of published papers by Selye and coworkers used to establish this thesis unfortunately contain a considerable amount of theorizing, making it difficult to discern which conclusions are justified by the experimental data. As far as participation of adrenal-hormone secretion in adaptation is concerned, the evidence is mainly indirect. The adrenal hypertrophy in response to many damaging agents has been noted by numerous workers in even the earliest reports, and has been reviewed by Tepperman, Engel, and Long (627). The histological changes have been interpreted to indicate increased adrenal activity. The associated findings in the "countershock phase" are undoubtedly similar to those following the injection of cortical extract. More direct evidence in support of adrenal participation in adaptation has come from other workers. Thus the finding of an increased secretion of a substance having cortical activity in the urine after various forms of trauma would indicate increased adrenal activity, especially since treatment with ACTH is followed by a similar increase in urinary cortin. Alterations in protein metabolism after trauma resulting in a negative nitrogen balance and an increased output of urinary

nitrogen would be in harmony with an increased adrenocortical secretion. Similarly, the changes induced in the cholesterol and ascorbic acid content of the adrenal glands following damage are most probably related to increased secretory activity. The most direct objection to the belief that the resistance developed by the animal to stress is due to the secondary secretion of adrenal hormones *per se* is that there is no evidence that any similar type of resistance has ever been produced by pretreatment with active cortical hormones *per se*. Furthermore, it has been shown that adrenalectomized animals maintained only on saline can develop a high degree of resistance to trauma like normal animals. However, the difference in the time taken to develop resistance and the gradual increase in trauma necessary to produce it in the adrenalectomized animal is in contrast to the response of the intact animal. Such resistant animals did not show any reduction in their tolerance to trauma over prolonged periods of time, so that no evidence of an exhaustion stage was observed (431). From a critical study of the experimental data it seems probable that the adrenal cortex maintains the physiological capacity of the body to resist in part any damaging agent but also to develop resistance if the insult is prolonged. Cortical secretion *per se* does not directly impart resistance, although an increased secretion may occur secondarily to stress. The speed, and probably the extent to which resistance can develop are apparently markedly influenced by the adrenal cortex.

Selye, from recent studies (556, 557, 561, 564-566, 568, 570), has suggested that during adaptation there may be an excess production of pituitary and adrenal hormones. The resulting endogenous hormone overproduction may give rise to certain cardiovascular, renal, and joint changes, which Selye has called "diseases of adaptation" (559). The experimental injection of large doses of DCA or pituitary extracts, or the inducement of the alarm reaction over a period of time, has led to a series of pathological conditions resembling nephrosclerosis, nephritis, periarteritis nodosa, arthritis and hypertension. Such a method involves the use of relatively large doses of hormone, but the reaction may be intensified by dietary measures, especially a high salt or protein intake. The altered metabolism of sodium chloride is of direct etiological importance, since certain acidifying salts may prevent the development of the lesions (559). The intimation that the etiology of similar diseases in the human is related to those produced by such experimental methods requires direct proof. Recently Harrison (213) has criticized this work, and in a repetition of Selye's experiments did not find that arthritic changes were necessarily caused by DCA treatment but rather that infection was intimately concerned with the cardiac and kidney lesions produced. Other workers (334a) have not found nephrosclerosis following DCA and salt treatment but did find

hypertrophy of the heart and kidneys. Hypertension was not found unless the kidneys were damaged by previous treatment. Changes in the cerebral arteries have been described in a boy with Addison's disease after DCA, salt, and cortical-extract therapy (155).

### XIII. Influence of Adrenal Cortex on Thymus, Lymph Nodes, and Blood Cells

That some relationship between the adrenal cortex and the thymus gland existed has been suspected for a number of years. Early observations on intact rats showed that adrenal hypertrophy and thymus atrophy followed various forms of stimulation, and Selye (551,552) has widely extended this field by his studies of the "alarm reaction." The reaction on the adrenals is mediated through the anterior pituitary gland and various observers have noted that injections of crude or highly purified pituitary ACTH causes adrenal enlargement associated with thymus atrophy and atrophy of lymph nodes (95,121,140,416,433,583). This effect of ACTH on the thymus and lymph gland does not take place in the absence of the adrenal glands.

In the adrenalectomized animal an increase in weight of the thymus gland and lymphoid hyperplasia occur (312,433,434,479,689). Conversely, cortical-extract treatment causes thymus atrophy (279). Adrenal steroids also active in this respect are corticosterone (681) and Compound E (281, 304). DCA has probably only a very slight action in causing thymus atrophy (281,553,681,687).

#### A. EFFECT OF ADRENAL CORTEX ON LYMPHOCYTES AND ANTIBODY PRODUCTION

Dougherty and White, in a series of recent fundamental papers, have correlated the changes in thymus and lymph glands with alterations in the circulatory lymphocytes. Associated with lymphoid tissue involution they observed a lymphopenia and an increase in serum globulin. Antibodies in the  $\gamma$ -globulin fraction which were concentrated in the lymphocytes were discharged into the blood. Following pituitary ACTH or cortical-extract administration, therefore, a specific defense mechanism of the body was enhanced (123a). The data from which these conclusions were made may be briefly reviewed. Some earlier workers had noted a lymphocytosis following adrenalectomy (91,724); the same change had been noted in Addison's disease (197) and these findings were confirmed by Dougherty and White (123). During studies on the alarm reaction, Harlow and Selye (209) noted that a harmful stimulus, besides causing thymus involution, also caused a delayed leucocytosis. Although the polymorphonuclear cells increased markedly, there was an associated relative lymphopenia. A lymphopenia is also found in the mouse after stress

(132a) or following hypertonic glucose given orally. Such an effect is not found after adrenalectomy (132). Dougherty and White (120,123) found that leucopenia and lymphopenia were consistently produced by the administration of 1.0 mg. purified ACTH in the mouse. The effect was most marked some 6-9 hours after the injection. The neutrophiles showed an increase and then a decrease. A similar effect was also produced in rats and rabbits. These findings have been confirmed in the rat and in the dog, although in the latter species subcutaneous injection of the ACTH is essential (478). The adrenalectomized mouse after similar treatment did not show any change in lymphocytes, although the neutrophiles were increased. The latter effect was therefore considered nonspecific. Cortical extract in a dose of 0.5 ml. produced the same blood changes in normal or adrenalectomized mice. Corticosterone was also active but DCA had no effect on lymphocytes. Histological studies showed the degeneration of lymph nodes and dissolution of lymphocytes to be present 3 hours after injection of ACTH (687). Continued daily injections of ACTH maintained the low level of blood lymphocytes although the total white cell count remained normal because of an increase in the polymorphonuclear cells (688). Yoffey and Baxter (713a) have described the regressive changes in lymphoid tissue of the rat following daily injections of ACTH for 1 month. Cases of Cushing's syndrome have been found to show a leucocytosis and a relative lymphocytopenia, whereas cases of Addison's disease exhibit the opposite change (107).

At the same time as the changes in lymphocytes take place a significant elevation of serum proteins occurs. Previously other workers using adrenalectomized cats and dogs had found that there occurred an increase in total serum protein. Despite the associated hemoconcentration, albumin was reduced, whereas globulin increased. Cortical extract or DCA induced hemodilution and a decrease in total serum protein and globulin but an increase in albumin. The albumin-globulin ratio therefore fell markedly during insufficiency and increased after treatment (236,348a,349). In untreated adrenalectomized mice in which hemoconcentration was prevented the serum protein behaved conversely, being reduced for at least 8 days postoperatively (687). Leathem (345), however, reporting paired feeding experiments in rats suggests that the lowered plasma albumin following adrenalectomy may be related to the reduced food consumption. White and Dougherty (689) further showed that single injections of ACTH in rats and rabbits consistently gave elevated serum protein.  $\beta$ - and  $\gamma$ -globulin fractions were significantly increased. Extracts of lymphocytes from lymphoid tissue on electrophoretic examination contained a protein component identical with  $\gamma$ -globulin of normal serum, indicating that lymphoid tissue is a storehouse of the globulin fraction. In another experiment cells

obtained from lymph nodes of immunized animals were found to contain high antibody titers when compared to other organs of the same animal (117). Following the single injection of adrenocortical extract into hyper-immunized rabbits, high antibody titers were produced within 6 hours. With repeated injection high titers were maintained for 2 weeks (118). Antibodies were apparently released from the lymphocytes, which show dissolution under pituitary-adrenal stimulation. Two toxic substances, benzene and potassium arsenite, also were found to induce antibody liberation indirectly through pituitary stimulation (119). From this work a pituitary-adrenal control over lymphoid tissue, which in turn affects blood lymphocytes, serum globulin, and antibody production, was postulated.

Since this work appeared there has been confirmation that the adrenal cortex exerts a control over blood lymphocytes and lymphoid tissue but a controversy exists over the release of  $\gamma$ -globulin and antibody production. Li and Reinhardt (356a) found that ACTH in the normal or hypophysectomized rat did not increase the antibody-containing globulin fractions. The albumin fraction was increased as was the albumin-globulin ratio. Eisen, Mayer, Moore, Farr, and Stoerk also found that cortical-extract treatment did not increase serum antibodies and  $\gamma$ -globulin in the rat (129a). They did however detect a transitory increase in preimmunized rabbits, in confirmation of Dougherty, Chase, and White. In humans the lymphocyte count has been shown to fall after stress and some difference in response has been noted in psychotic subjects (459a). Following treatment of two patients with ACTH, Forsham, Thorn, Prunty, and Hills could not demonstrate any increase in antibody titer or  $\gamma$ -globulins (154e). Similarly Herbert and de Vries (254a) did not detect any change in antibody level after ACTH treatment, although the lymphocyte and eosinophile count was markedly reduced by such treatment.

*Eosinophiles.* Following the injection of 25 mg. purified ACTH in man there appears to be a rapid decrease, with a maximum fall in 4 hours, of circulating eosinophiles as well as lymphocytes. Such changes were not found in patients with Addison's disease. However the same changes were evoked in these cases following the injection of 20 mg. 17-hydroxycorticosterone. This difference in eosinophile response to ACTH by normal persons and Addisonians has been suggested as a clinical diagnostic test by Forsham, Thorn, Prunty, and Hills (154e).

*Red Blood Cells.* Considerable evidence exists which indicates that there is a pituitary-adrenal influence on red blood cell production. This has been correlated and reviewed in a recent paper by White and Dougherty (688). It is pointed out that both in man and in experimental animals an increased secretion of the hormones of the anterior lobe of the pituitary or of the hormones of the adrenal cortex results in polycythemia. Conversely, pituitary or adrenal insufficiency is associated with anemia.

Experimentally, ACTH injections, as well as causing the previously described changes in leucocytes, were followed by a rise in red-cell count and blood hemoglobin. Conversely, adrenalectomized rats showed a significant decrease in hemoglobin and red-cell count, while possessing normal whole-blood specific gravity. Adrenalectomized mice did not show an increased red count although they showed a decreased plasma volume (688). The effect of adrenalectomy and treatment with adrenal steroids has been studied on red-cell respiratory metabolism in the rat. Removal of the adrenals produced a decrease of 73% in oxygen uptake. Treatment of the rats with cortical extract, DCA, or saline caused a return of oxygen uptake to normal. No changes in reticulocyte counts were observed after adrenalectomy or treatment (190a).

#### B. MALIGNANT DISEASE OF LYMPHOID TISSUE AND LEUKEMIA

The dissolution of lymphoid tissue which takes place after injections of cortical extract or secondarily to ACTH and various forms of treatment which stimulate the anterior pituitary has been investigated in relation to malignant changes in the blood cells and lymphoid tissue. It seemed possible that ACTH might have therapeutic value in such conditions as leukemia, and some experimental work in this field has been reported. The similarity in the response of lymphoid tissue to irradiation, urethan injections, and ACTH have been noted by a number of workers. Leblond and Segal (346a) found generalized involution of lymphoid structures following x-radiation in normal but not in adrenalectomized rats. In the latter case, however, lymphoid changes were produced in the irradiated area. Other workers have found that higher dosage of x-radiation will cause lymphoid involution in the adrenalectomized animal. It seems therefore that small dosage causes an effect through indirect liberation of ACTH, whereas larger dosage causes direct changes in the lymphoid tissue (123a,447a). The action of injected ethyl carbamate (urethan) is of interest since Hawkins and Murphy in 1925 (248a) commented on the similarity of its effects on lymphoid tissue with those caused by x rays. A large number of reports have now confirmed that of Patterson, Thomas, Haddow, and Watkinson (447b) that urethan caused a temporary fall in white blood cells and a diminution in the size of the spleen and lymph nodes in human leukemia and that the effect was similar to deep x-ray therapy. Leukemia in the mouse and rat have subsequently been found to be favorably influenced by urethan (133a,333a,344a,421c,677a). Murphy and Sturm (421c) have noted that the adrenalectomized rat showed an increased susceptibility to transplanted lymphatic leukemia (421a,598b), and that treatment with cortical extract, DCA, or ACTH had an inhibiting effect on its development (421b). Since urethan caused adrenal enlargement and lymphoid atrophy it was thought possible that its action

was through stimulation of the anterior pituitary to release ACTH. Other workers have also tested the action of cortical extract or adrenal steroids on spontaneous or transplantable lymphoid leukemia in mice (252a,344b). A favorable response on circulating lymphocytes and lymph nodes was noted so that the malignant cell apparently responded to treatment in a similar manner to normal lymphoid cells.

#### XIV. Alterations in Cholesterol and Ascorbic Acid Content of Adrenal Cortex

That histological changes take place in the adrenal glands after damage to the organism has been noted by many observers; these reports have been recently reviewed (531a,559). The changes in stainable lipide which occur have suggested that chemical alterations in the gland also take place. In 1928 Crema (95a) found the cholesterol content of the adrenals to be depleted in guinea pigs after burns. The same change is also found in rats (531a). In rabbits, Clark and Rossiter (67a) showed that burns are followed by a fall in the ascorbic acid content of the adrenals but not of the liver. Tourniquet shock in rats was shown by Popjak to be followed by depletion of adrenal cholesterol. Staining reactions indicated that hypertrophied adrenals contained an increased hormone content and it was suggested that cholesterol was the precursor of the hormones (461a). Various forms of stress, such as cold, low oxygen pressure (348), total body irradiation (122,447a), administration of large doses of water (173), or feeding carbohydrate (1) reduce the cholesterol content of the adrenals and may cause changes in stainable lipides in the cortex (668c).

Sayers, Long, White, and associates have conducted an extensive systematic study of the chemical changes occurring in the adrenals and have correlated them with adrenal function. In 1943 Sayers, Sayers, White, and Long (531c) showed that a single dose of 2 mg. purified ACTH was followed in 3 hours by a reduction of approximately 50% in the cholesterol content of immature-rat adrenals. If the injections were continued for 3 days the cholesterol concentration increased over that found in control animals. The observations were confirmed by Carreyett, Golla, and Reiss (64), who also found that gonadotrophin from pregnant mare serum had an opposite effect to ACTH on adrenal cholesterol. In later papers (531,531b) Sayers *et al.* found also that ACTH reduces the adrenal ascorbic acid content. Following a single dose the level of ascorbic acid was reduced to two-thirds in 20 minutes and to half its normal value in 1 hour. A return to normal had occurred in 9 hours. Changes in the cholesterol content were less rapid, since the maximum fall occurred at 3 hours and restoration was not complete in 9 hours. In extensive experiments on nonfatal hemorrhage in rats it was found that similar changes occurred in the adrenal ascorbic acid and cholesterol content (531a). Hypophysec-

tomized animals did not show such changes. The cholesterol content of the liver and brain was unchanged following hemorrhage. The ascorbic acid content of the liver rose during nonfatal hemorrhage, but brain levels were unaffected. Serum cholesterol fell during shock due to hemorrhage, whereas the serum ascorbic acid increased. Since such alterations did not occur in the absence of the pituitary gland and since ACTH induces the same effect as hemorrhage, a pituitary control over the adrenal metabolism seems well established. Many substances have now been found to reduce the adrenal ascorbic acid. These include benzene, estrogens, ether, chloroform, insulin, diphtheria toxin, tetanus toxin, anoxia, active infections, atropine, nicotine, histamine, cold, dibenamine, and intraperitoneal glucose. Detailed references to the substances listed may be found in the review by Sayers and Sayers (530b). The mechanism by which the pituitary is stimulated to release ACTH is not yet established, although Long and Fry (366) have noted that adrenaline caused a reduction in adrenal cholesterol and ascorbic acid, but this effect was abolished by hypophysectomy. Sayers and Sayers (530,530a,b) found that the expected decrease in adrenal ascorbic acid following various forms of stress (cold, heat, injections of histamine, adrenaline, or killed typhoid bacilli) did not take place when the animal was pretreated with cortical hormone, 17 - hydroxycorticosterone, 17 - hydroxy - 11 - dehydrocorticosterone, desoxycorticosterone, and progesterone which were assigned estimated potency ratings of 4, 4, 1, 0.5, and 0.02, respectively. They have interpreted their results to mean that the anterior pituitary elaborates ACTH at a rate inversely proportional to the concentration of cortical hormones in the body fluids. A large number of workers have confirmed the changes originally described as occurring in adrenal ascorbic acid or cholesterol following various forms of injury or stress (208a,b,348,374b,c,d,628a). Alterations in the adrenal cholesterol or ascorbic acid of the hypophysectomized rat may be used as the basis of a sensitive method for assaying pituitary ACTH (531c,419,532). The alterations in adrenal cholesterol and ascorbic acid associated with changes in the amount of Sudanophilic substance in the gland would seem likely to be related to hormone formation. The compound containing a vitamin C - steroid complex isolated from adrenals by Lowenstein and Zwemer (374a) has considerable theoretical interest in relation to the above work. However until confirmation of this report is available it is considered unwise to speculate on its significance.

#### XV. Cortin Content of Adrenal Venous Blood

An indication of adrenal secretory activity and subsequent metabolism has been furnished by studies reported by Vogt (668a). Blood removed from the adrenal vein of various animals has been assayed for cortin by

the Selye-Schenker cold-survival test. Whereas arterial or ordinary venous blood did not contain activity, the adrenal venous blood was highly active. From comparative assay it was found that 1 ml. plasma from adrenal blood was up to ten times more active than cortical extract, equivalent to 1 g. adrenal tissue. With the highest values obtained, 1 ml. plasma would be equivalent to approximately 0.15 ml. cortical extract. The average output/min.kg. from one adrenal was found to be equal to the cortical activity of 0.6 g. adrenal tissue. The daily output of a 10-kg. dog was therefore calculated to be equal to 230 ml. cortical extract. In a subsequent paper (668b) it was found that adrenaline caused an increased output of cortin into the adrenal blood. Such an effect was immediate and direct, being independent of the pituitary. In studies on the fate of the secreted cortin it was found that once it reached the general circulation loss of activity was rapid. Inactivation still occurred rapidly even though the viscera, liver, and kidney were removed (668a). Butcher (62a) has used autotransplantation of the adrenals into various organs to determine which tissues most rapidly destroy cortical secretion. There appeared, however, to be no difference in the inactivating effect of liver, spleen, or kidney. Vogt (668d) has shown that plasma obtained from adrenal blood did not contain significant amounts of ascorbic acid even though cortical activity was present. These observations make it seem unlikely that the active cortical hormones are secreted as a steroid-ascorbic complex as has been suggested (374a).

## XVI. Miscellaneous Functions

### A. EFFECT OF ADRENALS ON BONES AND TEETH

That some interrelationship between the adrenals and the parathyroid glands may exist has been suggested by some of the early observations of Rogoff (500,505). The influence which the adrenals may have on bone, however, requires further study. Adrenalectomy of the growing rat results in a decreased width of the epiphyseal cartilage, but this is probably related to a reduced food intake (276,712). A later increase in width of the cartilage has been suggested as related to a secondary increased activity of the pituitary gland (712). The adrenalectomized rat maintained on salt has been reported to show a greater increase in serum calcium and in calcium excretion than the intact animal after injection of parathyroid extract (467). Williams and Watson (693) have shown that cortical extract, corticosterone, or Compound E cause a reduction in the phosphatase of the diaphyses and epiphyses of the rat. The amorphous fraction had no effect on bone phosphatase, whereas DCA, in distinction to other steroids, caused an increase. This action was compared to that of parathyroid extract. Clinically, cortical extract has been described as having a beneficial effect in Paget's disease and in chronic arthritis, by Berman

(26,27) and Watson (675,676). Adrenalectomy in the rat has been found by Schour and Rogoff (535) to cause a disturbance in the calcification of dentine of the teeth and characteristic histological changes. Becks, Simpson, Li, and Evans have found bone changes associated with the growth inhibition which follows treatment with highly purified ACTH (22). Albright (2b,c) has presented clinical evidence that osteoporosis may be influenced in part by adrenocortical hormones.

#### B. EFFECT OF ADRENALS ON HAIR

From the clinical observations that hirsutism is frequently associated with adrenal tumors, it might be expected that experimental study would indicate some definite interrelationship between the adrenals and hair growth. Butcher (61), however, found that in the adrenalectomized rat hair growth occurred earlier than in intact controls. Furthermore, a condition of underfeeding, which is associated with adrenal hypertrophy in the rat, resulted in retarded hair growth which continued until the adrenals were removed (62). These results are the opposite to what would be expected from clinical observations. However, no one has studied the effects on hair growth of adrenal steroids *per se*. Stein and Wertheimer (594) noted a transient increase in the rate of hair loss in adrenalectomized rats, associated with skin symptoms. These also occurred in medullectomized animals and could be prevented by adrenaline, but not by cortical extract or DCA. Hair coat changes in adrenalectomized dogs, however, apparently respond at least in part to DCA treatment (620).

#### C. EFFECT OF ADRENALS ON SKIN PIGMENTATION

Increased pigmentation of regions of the skin exposed to light or pressure is one of the cardinal signs of Addison's disease and is usually present to a greater or lesser extent. Following treatment, some modification of the intensity of the pigmentation may occur (414a). These changes seem peculiar to humans, since corresponding alterations have not been reported in adrenalectomized animals. It is possible that the adrenal medulla may be involved in such pigmentation. Spoor and Ralli (589) have described changes in skin melanin formation, attributed to an adrenal influence, and the additional effects of certain deficient diets have been noted (471). The adrenals do not apparently influence the pigmentation following administration of certain sex hormones (105).

### XVII. Adrenal-Gonad Relationship

The functional relationship between the adrenal cortex and the gonads has been studied by numerous workers on humans and experimental animals. The reciprocal interrelationship and effects on secondary sex characteristics have been greatly expanded in recent years, and Parkes has

recently published an extensive review of 412 papers on the subject, (442). It is beyond the scope of this chapter to consider this field in any detail, but for the sake of completeness a brief summary has been constructed, chiefly from Parkes' review.

A connection between the adrenals and gonads has been suggested by the observations of sex dimorphism of the adrenals, generally a size difference or histological distinctions, in a number of species of animals. Distinct changes in the adrenals may be associated with the reproductive cycle and also occur following removal of the gonads. Gonadal hormones also have a direct effect on the adrenals, tending to correct the changes associated with hypogonadal activity. Removal of the adrenals has in general a suppressing effect on gonad activity, which is corrected by cortical-extract treatment. That the gonads may possess cortinlike activity has been previously noted in survival experiments. Progesterone is active in such cases. The condition of the activity of the ovaries, pregnancy, or pseudopregnancy thus markedly influence the effects of adrenalectomy. Experimentally, there seems to be definite evidence that under certain conditions the adrenals may secrete substances having sex hormone activity. Androgens, as reflected in secondary sex organ changes, may be produced by the adrenals after castration in young animals or in more conclusive experiments by the adrenals of male or female rats stimulated by ACTH. Some evidence is found that an estrogen-like effect may occur secondary to adrenal secretion in female animals. Removal of the ovaries at birth in mice may be followed by cyclic changes in the reproductive organs and the appearance of mammary cancer in certain susceptible strains. Abnormal hormone production in the adrenal glands apparently takes place and these glands may show gross abnormality and tumor formation. This evidence indicates that in animals the adrenals under certain circumstances may produce estrogens or androgens in physiologically effective amounts. Spontaneous dysfunction of the adrenals on a scale adequate to disturb the reproductive organs and secondary sexual character is found only in man. The adrenogenital syndrome and certain types of virilism are examples which are discussed in other chapters of this volume. Certain of the adrenal steroids may *per se* exhibit androgenic, estrogenic, progestational, anesthetic, or other properties, and a complete list of pharmacological data may be found in Selye's encyclopedia (558). This book contains descriptions of the extraction of sex hormone activity from adrenal glands, or adrenal tumors, and the various secretory products isolated from the urine.

#### LACTATION

Closely dependent on the interrelationship of the adrenal cortex and the gonads is the influence of the adrenals on lactation. Because of the com-

plex hormonal control of lactation itself, and of the secondary effects of adrenal influence, the subject contains a considerable number of conflicting reports. Furthermore, there is some evidence that a specific hormone affecting lactation may be produced by the adrenal. Nelson (423) and Nelson and Gaunt (424) have published extensive reviews on this subject. Adrenalectomy has been shown by Carr (63) and Gaunt (169) to interrupt or reduce lactation in rats even though they were prevented from showing marked symptoms of insufficiency by extract treatment. However, Swingle and Pfiffner (616) showed that cortical extract would maintain lactation in normal bitches. Gaunt and Tobin (182) later found that, if sufficient dosage of cortical extract was given, adrenalectomized rats could lactate normally. The dose required was at least twice that necessary for normal maintenance. Treatment with salt or low doses of cortical extract had some effect, but the former alone was never entirely adequate. DCA treatment did not allow normal lactation to take place, although improvement occurred, as with salt treatment, since the animal's general condition was restored (94a,b,171,174). Gaunt, Eversole, and Kendall (174) found cortical extract to act as a complete replacement therapy in lactating adrenalectomized rats. Compound E in a daily dose of 1.0 mg. was also completely effective. Dehydrocorticosterone was beneficial, but rather less so than Compound E. The amorphous fraction had little effect. Gomez and Turner (189,190) and Nelson and Gaunt (425,426) have studied lactation in hypophysectomized guinea pigs. Whereas neither prolactin nor cortical extract will initiate or maintain lactation alone, the two extracts given together were effective (189,426). Compound E (427) or ACTH may be successfully substituted for cortical extract (190). Salt therapy and prolactin together was also effective (425). In 1933 Brownell, Lockwood, and Hartman (53) presented evidence that whereas adrenalectomized rats maintained on cortical extract would not lactate they would do so if a separate adrenal fraction termed "cortilactin" were also given. Further work (588) showed that although normal lactation would follow three times the cortin maintenance dose, the "cortilactin" fraction given with a single maintenance dose would also allow milk secretion. "Cortilactin" was separated and prepared by one of the processes used for obtaining prolactin, and it assayed at one-tenth the activity of the purified pituitary hormone by the crop gland stimulation method. The adrenal fraction had no effect on carbohydrate metabolism. The status of "cortilactin" at present appears unsettled.

#### XVIII. Therapeutic Action of Compound E (Cortisone)

The preliminary report on the effect of 17-hydroxy-11-dehydrocorticosterone (compound E) and pituitary ACTH on rheumatoid arthritis by Hench, Kendall, Slocumb, and Polley (253a) has been received with

great interest. Undoubtedly a rapid and further expansion of these fundamental observations will follow in the immediate future, but at this moment the above contribution is the only one published in a scientific journal. The findings presented in this paper may be summarized. The rationale for adrenal steroid therapy in rheumatoid arthritis was developed by Hench after prolonged observations on patients suffering from this disease. Due to remissions of the disease which occurred during pregnancy, or were associated with jaundice, it was postulated that the course of the disease could be profoundly influenced by phenomena caused by some basic biochemical change. The temporary improvement in the arthritic resulting from anesthesia or surgical operation suggested that secondary stimulation of the adrenal cortex might be involved. The synthesis and preparation of small quantities of 17-hydroxy-11-dehydrocorticosterone (compound E) by the cooperative effort of the research chemists of Merck & Co. Inc. and Kendall and his associates (253a) provided material for clinical tests. Compound E was first used, but was later replaced by compound E-acetate. (In order to avoid confusion with Vitamin E the name cortisone has been suggested instead of compound E-acetate.)

The results of treatment of 14 cases of severe chronic polyarticular rheumatoid arthritis with cortisone were dramatic. Within a few days marked symptomatic improvement was noted. Reduction of the stiffness of muscles and joints, loosening of articular aching or pain and tenderness and improvement of articular and muscular function was apparent. Improved appetite and muscular strength, increase in weight, and sense of well-being or euphoria was also noted. The sedimentation rate was decreased following treatment, and elevated serum globulin values when present were lowered and normal A/G ratios established. Urinary 17-ketosteroids were reduced, corticosteroids in the urine elevated, and the anaemia improved by cortisone. The optimal dose of cortisone employed was 100 mg. daily given as a single injection of a fine crystal suspension in 4 ml. of saline. Doses of 25 or 50 mg. were usually inadequate. Cessation of treatment led in most cases to a return of symptoms and joint involvement and a prompt rise in sedimentation rate. Prolonged treatment (some 7 months) in one case has led to endocrine disturbances resembling those found in Cushing's syndrome. Dehydrocorticosterone was given to one patient in a dose of 100 mg. daily, but did not cause any improvement. The same patient later responded satisfactorily to cortisone injections. Two patients with rheumatoid arthritis were also treated by daily intramuscular injections of 100 mg. of pituitary ACTH prepared by Armour and Co. Marked clinical improvement essentially similar to that found with cortisone was noted. A return of symptoms took place on cessation of treatment.

Encouraging results were noted following cortisone treatment of one case of severe lupus erythematosus and polyarthritis and one case of acute rheumatic fever and therapy was still in progress. Extensive studies on these and other related conditions are no doubt in progress following the discovery and stimulus given by the above studies and the published results will be awaited with interest. At present there seems no obvious physiological explanation of the dramatic results of cortisone on the conditions described, especially if dehydrocorticosterone should prove to be inactive. It is of interest to note that Watson (676) some years ago detected some symptomatic clinical improvement in patients with chronic arthritis following the injection of 3 to 5 cc. of cortical extract weekly, while studying the effect of such treatment on serum phosphatase. There is no evidence available to indicate that a failure of adrenal or pituitary function is the etiological factor of the diseases that respond to cortisone therapy, neither is there any evidence to indicate what metabolic processes are primarily affected to induce the marked amelioration of symptoms. Further improvement in the supply of the present synthetic production of cortisone, new methods of synthesis or the discovery of other compounds with a cortisone-like effect will be eagerly awaited so that adequate material may be available to thoroughly study this important new field of adrenocortical influence.

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## CHAPTER IV

### The Chemistry and Physiology of the Thyroid Hormone

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In this section the thyroid will be considered not as an isolated gland but as an integral part of the total endocrine balance. The thyroid acts as a central distributing station for iodine metabolism, and in this way influences the total iodine balance of the organism. It also supplies the tissues with a specific hormone which has characteristic chemical and biological properties. This substance is distributed throughout the various tissue cells of the body and may be traced by its inherent iodine. Much of the discussion which follows will be based upon this endocrine function of iodine (401).

The wide-spread distribution of such organically bound iodine suggests that the thyroid is fully as important physiologically as the pituitary gland,

although the former plays its role in a humbler sphere. In fact, after complete removal of the thyroid the iodine content of other glands, like the pituitary and ovary, falls; and simultaneously such glands fail to function normally. Possibly the same phenomenon, with reverse emphasis as to iodine, occurs in hyperthyroid states. In short, if the pituitary is viewed as "the leader of the endocrine orchestra" (89), perhaps the thyroid may be regarded as the first violin.

### I. Thyroid Structure and Development

It would appear from the work of Anton Dohrn (98,99) that the thyroid originally belonged to the alimentary tract. This work amplified the pioneer observations of Müller (331) and was confirmed by Marine (289) and Leach (248). Whatever alimentary function it may once have had has disappeared in the course of ages; its main function now is to elaborate and control the supply of an essential hormone. Under normal conditions the concentration of this essential substance is maintained within close limits, as hereinafter described. There is still some question how early in the evolutionary scale a physiologically active organ can be detected. The first evidence of an iodine-fixing organ which appears valid occurs in the cyclostomes, primitive fish which are like the lampreys. Below this evolutionary level the "endostyle" is perhaps the homologue of the thyroid; but there is no evidence that it fixes iodine, or possesses endocrine potency.

It has been said that, with the exception of the sex glands, the thyroid is the oldest of the endocrines [Engelbach (163)]. It is clear that the thyroid originated as an out-pocketing of cells from the primitive endoderm. This evagination of the embryonic pharynx subsequently gave rise to the anlage of the adult gland, which contained characteristic epithelial plates and bands (cf. 16). Gorbman (155) has found that iodine is metabolized specifically only in the cyclostomes and higher vertebrates. In the "endostyle" of these organisms, a single epithelial layer has the property of concentrating administered radioiodine, as shown in Fig. 1. The lower forms of chordates store no iodine. Thus, at a time when sea squirts have a definite pituitary anlage, one can find no physiological evidence that they possess a gland specifically connected with iodine metabolism. The simpler *Amphioxus* possesses physiologic evidence of neither pituitary nor thyroid endocrine function.

During embryonic development, the tubular connection of the primitive thyroid tissue with the pharynx retains its attachment for a time as the glandular primordium extends downward. The resulting stalk is called the thyroglossal duct. In the human embryo this structure persists normally up to about the fifth week. The pharyngeal region (*tuberculum impar*) from which it takes origin later becomes the back of the tongue. After the fifth

week, however, the lumen of the duct becomes occluded and shortly afterward disappears in most cases, so that only the foramen cecum remains in the fetus, a tiny dimple on the back of the tongue. This relic, therefore,

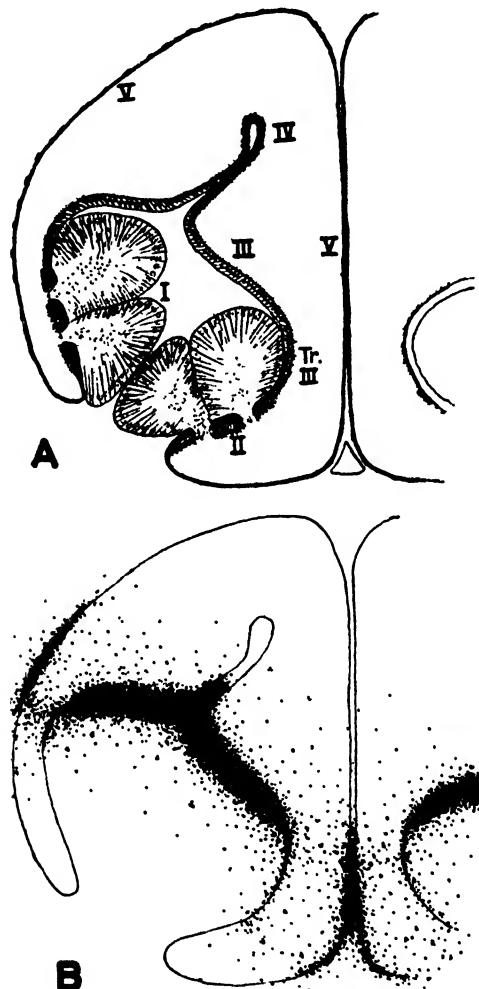


FIG. 1.—The dawn of the thyroid's specificity for iodine is mirrored in the "endostyle" of cyclostomes, as shown by Gorbman and Creaser (*J. Exptl. Zool.* **89**, 395, 1942). A. Section of an anterior chamber of the endostyle. B. Radioautograph of this section projected on a tracing of itself.

documents man's relation to certain primitive fish in which this pit still leads into the thyroid itself. The interrelations of these anlage are pictured in Fig. 2.

These phylogenetic maneuvers sometimes miscarry. Consequently the clinician sees cases of embryological arrest or maldevelopment, e.g., sometimes the migration of the thyroid anlage fails, and all of the thyroid tissue in the adult resides in a lump at the back of the tongue which is called a "lingual thyroid." In other cases the thyroglossal duct fails to atrophy com-

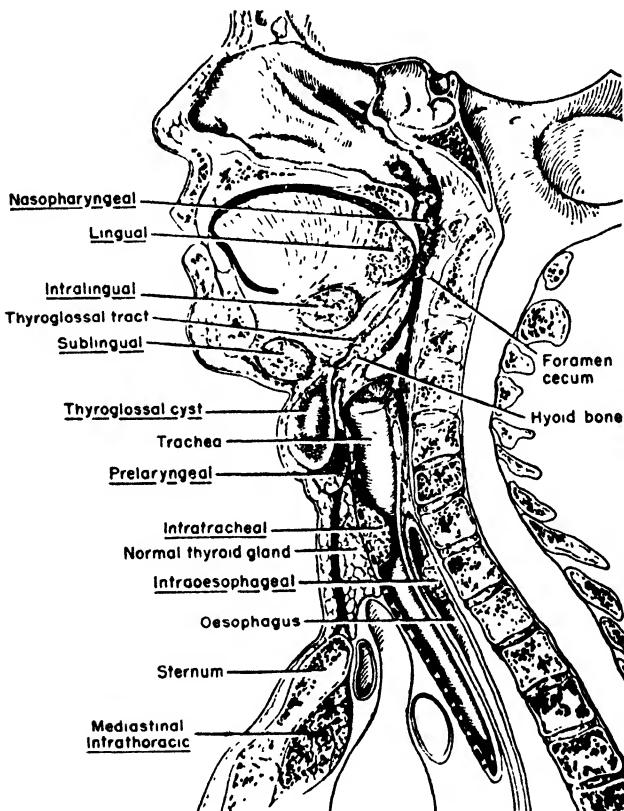


FIG. 2.—The foramen cecum of the tongue, the rare patent thyroglossal duct and masses of aberrant thyroid tissue in the adult, all document the story of embryonic thyroidal development. Locations of Aberrant Thyroids (after Rienhoff, Lewis' *Practice of Surgery*, W. F. Pryor Company, Inc. Cited by Means in *Thyroid and Its Diseases*, J. B. Lippincott Company, Philadelphia, 1937.)

pletely, and its remnants may require surgical intervention in the adult. Masses of thyroid tissue may be found in the adult's neck, far out toward the shoulder, or even in the mediastinum, as shown in Fig. 2. Occasionally the surgical removal of such an aberrant mass may deprive a patient of all his functioning thyroid tissue. Such aberrant tissue, however, must not be confused with metastases from a cancer in the main thyroid mass.

Norris (342) and Wölfler (535), in addition to the "medial" anlage, described two lateral contributions, each from the fourth pharyngeal pouch. These masses of "ultimobranchial tissue" finally become fused with the main mass. Certain cystic enlargements in hypoactive thyroids in adult men perhaps may be derived from these ultimobranchial sources (513). Such tissues contain stratified squamous epithelium. They are found in older thyroids and suggest potential neoplasia. Thus the embryologic history of the gland may be related to the ultimate development of cancer in the adult.

In the cartilaginous fishes (elasmobranchs) the thyroid is situated near the anterior end of the ventral aorta. The follicles of the gland, which varies greatly in shape, contain cells of high cuboidal or columnar character. In the bony fishes (teleosts) the gland is diffuse, consisting of scattered follicles along the ventral aorta between the first and the third gill arches. The follicular cells are cuboidal. Goitrous enlargements of these masses have been described by Marine and Lenhart (294). The thyroid first becomes a paired organ in the Amphibia; but in reptiles it remains a single gland situated above the pericardium. In some birds the gland comprises two ovoid masses in close relation to the internal jugular vein and the carotid artery of either side. In mammals, however, the paired gland tends to be applied closely to the trachea, and often the two lateral lobes are connected by an isthmus molded over the anterior and lateral aspects of the trachea. In human patients this feature is of considerable diagnostic interest.

The early morphogenesis of the human thyroid has been studied in detail by Norris (343). It can be detected as early as the third week (1.5 mm. embryo) as a diverticulum arising from the floor of the pharynx between the first and the second pharyngeal grooves; it develops laterally so that as early as the 5-mm. stage the bi-lobed nature of the future gland is evident. The stalk of the bulbous structure is first hollow, but later solid, and remnants of it persist even as late as the 15-mm. embryo, but the connection of the stalk with the floor of the pharynx is ordinarily lost at about the 7-mm. stage. In the beginning, the parenchyma of the gland is composed of fenestrated epithelial plates arranged longitudinally. From these cords of cells the first follicles develop at about the 24-mm. stage. These "primary" follicles are originally devoid of colloid. At about 65 mm., a secondary formation of follicles develops which continues until the 158-mm. size is reached. Thereafter there is simply an increase in the size of the pre-existing structures.

There is some question when the colloid first appears in the fetal thyroid. Keene and Hewer (228) found traces of colloid at the eleventh week of intrauterine life; and Elkes (116) demonstrated colloid at 4½ months. Certainly colloid is present in the later months of fetal life (332); and at birth well-formed follicles containing ample stores of colloid are present normally. By means of radioactive iodide, Chapman *et al.* (67) studied the function of

the human fetal thyroid. The subjects were women whose pregnancy was terminated surgically because of danger to health. Twelve to forty-eight hours before operation, radioactive iodine was administered to the mothers. Between the ages of 14 and 32 weeks, the fetuses showed increasing uptakes of radioactivity. Thus physiological function does not appear usually in the fetus until after follicles containing typical colloid are found, namely, after the third month.

In the rat fetus iodine storage begins in the thyroid between the 18th and 19th day of fetal life (156), i.e., nine-tenths of the gestation period has passed before there is evidence of characteristic thyroid metabolism. Corresponding data for human fetuses (298) suggest that iodine storage begins at about the 28th week of intrauterine life. In pigs and sheep the date lies between the second and fourth month of gestation (129,208). Probably these figures depend upon the necessity for the fetal thyroid's activity. For example, in Cannon's experiment with the 10 weeks pregnant thyroidectomized bitch (56), apparently precocious functioning by the fetal thyroid preserved the mother's health until after her delivery. This fetal effect has been amply confirmed in cows (363,476) and in bitches (102,363, 476). Similarly, hyperplasia is the rule in the fetal thyroid encountered in endemic cretinoid areas. By the time the cretinoid child is born the thyroid has assumed the proportions of a true goiter. Also the use of goitrogenic agents in the mother may perhaps lead to precocious development of the thyroid of the fetus (154). More data are needed to describe this fetal activity in terms of the elaboration of thyroxine. The present data are largely in terms of iodine, which may not possess endocrine potency. When the pregnant mouse is treated with thiouracil, the fetal thyroids are affected seriously. The normal configuration of follicles fails to develop after the 16th day and no colloid is laid down (225,451). After mid-term in goats the fetal thyroids become greatly enlarged when the mothers are treated with thiouracil. In fetal rats, Barnett (27) found serious interference with the development of the central nervous system, not because of the thiouracil *per se* but because of hypothyroidism. This finding has an important bearing upon the prognosis in cretinism as contrasted with juvenile myxedema.

In young animals the thyroid is active and tends to develop faster than the general body tissues. At puberty, approximately, the greatest relative mass of thyroid tissue is achieved, and there is involution in later life (302). In the normal human adult, given sufficient iodine, the thyroid weighs about 25 g. when fresh or between 4 and 5 g. when dried. The concept of normal weight, however, varies in different localities. Oswald (348), in Switzerland, recorded the weights of glands which appeared normal on microscopic examination. The highest figure was approximately 95 g.! Ob-

viously the individual had enlarged his hormone factory in order to utilize effectively the meager concentration of iodine available.

In slender individuals, especially in young women, the thyroid is readily discernible as a symmetrical prominence of the neck on either side of the larynx. Although the gland in crude and undissected form resembles a shield (524), this resemblance is less apparent on close examination. Characteristically, there are two main lateral lobes closely applied to the larynx

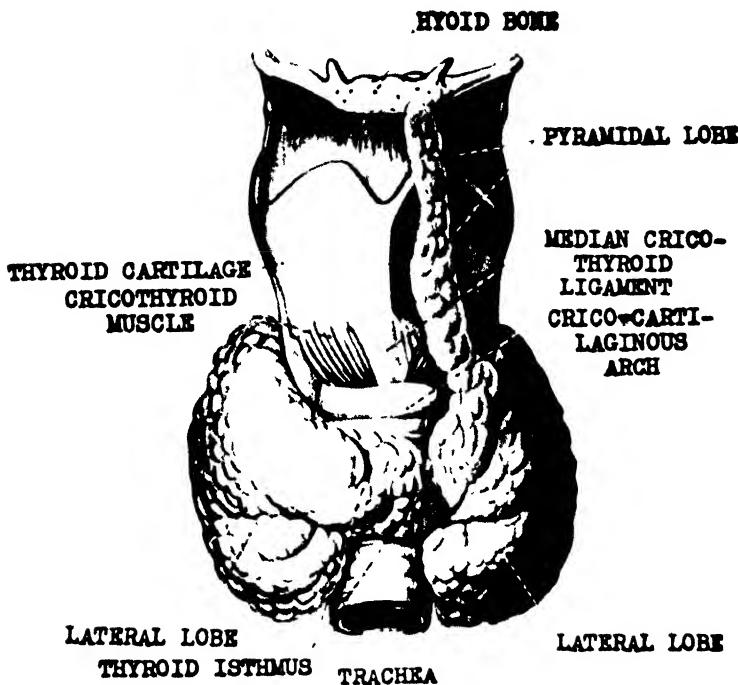


FIG. 3.—The gross anatomy of the thyroid (From *Atlas of Human Anatomy Vol. II*, by Sobotta, G. E. Stechert & Co., New York, 1933).

(Fig. 3), connected near their lower poles by a small isthmus which stretches across the front of the trachea near its upper end. From the isthmus a small tongue of tissue may extend upward over the face of the cricoid cartilage of the larynx. This pyramidal lobe is inconspicuous ordinarily, but during diffuse hyperplasia of the gland it may enlarge so that it is palpable through the skin.

The thyroid is exceptionally well supplied with circulation. Indeed, an amount of blood equivalent to the entire bodily content traverses the gland in an hour. Similar figures for the lungs and kidneys are one and five minutes

respectively, but the thyroid amounts to only 0.03 % of the total body mass. During cyclic variations in thyroid function this rich supply of blood may undergo alterations. Indeed, it has been suggested (341) that changes in the circulatory rate may alter the supply of hormone. In Graves' disease a tremendous increase of this blood supply causes the palpable thrill and audible bruit which are classic signs of the disturbance.

The follicles are closed cavities without direct structural communication to the outside,  $20 \mu$  to nearly 1 mm. in diameter. The typical follicle is about  $160 \mu$  long and roughly ovoid, although occasionally compressed; it is lined by a single layer of epithelial cells resting on a support of fine connective tissue fibrils, but there is no true basement-membrane. The cells are normally cuboidal, but may become columnar under forced activity. After appropriate staining, and under very high magnification, droplets of eosinophilic colloid can be distinguished within each cell during the so-called "active" phase. This colloid can be withdrawn by microdissection (391). Williams (528) has pictured the accumulation of these fine droplets into a large globule which then is budded off at the inner face of the follicular cell (*cf.* the secretion of a mucous globule or of a milk globule).

The typical epithelial cell has a spherical nucleus with prominent nucleoli, and mitochondrial filaments 0.2 to  $0.4 \mu$  by about  $0.2 \mu$ . These tend to be arranged parallel to the long axis of the cell, and are most frequent on the side nearer the lumen of the follicle. These mitochondria are particularly conspicuous when the gland is stimulated to forced activity. With appropriate staining reactions, Cowdry (82) and Maria Krogh (242) demonstrated a prominent Golgi apparatus which, when the cells are stimulated with thyrotropic hormone, undergoes characteristic changes, and near which droplets of colloid appear (277). The Golgi apparatus itself then lies between the nucleus of the cell and the lumen of the follicle. Under certain circumstances a "reversal" of the polarity of the cell has been described (401).

It is unlikely that the parenchyma of the gland is supplied with nerve fibers (340). The smaller blood vessels, however, receive fibers from both branches of the autonomic system. The sympathetic fibers are derived from the cervical ganglia; the parasympathetic fibers from the ganglion nodosum of the vagus nerve. These fibers enter the gland along the walls of blood vessels in the former case and in branches of the laryngeal nerves in the latter case.

Under conditions when more hormone is demanded by the organism it may be necessary to expand the standing equipment of each of the follicles. This expansion is largely motivated through the thyrotropic hormone of the anterior pituitary. The first visible anatomical response is a hypertrophy of each cell, the depth of which may double. Simultaneously, the water

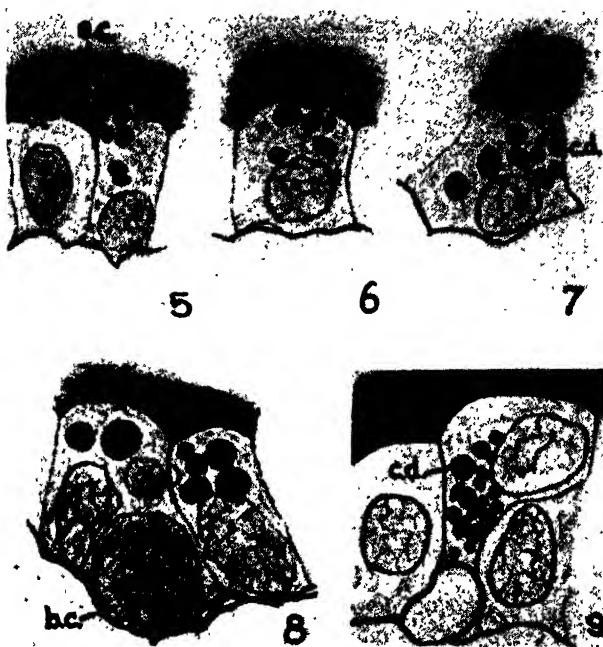


FIG. 4. When excretion of hormone into the blood stream occurs directly from the follicular cell, instead of "endoexcretion" into the follicular lacuna, this reversal of cell polarity is indicated by changes in the intracellular morphology. Such changes involve the nucleus, the Golgi apparatus, mitochondria, and eosinophilic droplets. Parts 5 to 9 above, are drawings made with the help of the camera lucida of frozen-dried-denatured sections of thyroid gland, stained with Anilin Blue-Orange G (90 X objective, 15 X ocular, table level). All cells are with the apical pole up.

5. Two thyroid cells of a rat 30 minutes after the injection of 3 units of thyrotropic factor. Both cells are excreting colloid droplets into the lumen. Three colloid droplets surrounded by a cytoplasmic film are seen in the lumen after the rupture of all connections with the cell.

6 and 7. Thyroid cells 60 minutes after the injection of 1 unit of thyrotropic factor. Two different stages of excretion are seen.

8. Thyroid cells 3 hours after the injection of 10 units of thyrotropic factor. Cytoplasm is filled with colloid. One cell has two large droplets of basal dilute colloid. No indications of excretion are present.

9. Thyroid cells 22 hours after the injection of the thyrotropic factor. One cell shows a complete inversion of its morphological polarity.

c. d., colloid droplets; e. c., excreted colloid droplets; b. c., basal colloid. (From "Intracellular colloid in the initial stages of thyroid activation" by E. De Robertis, *Anat. Record* 84, 134-135, 1942).

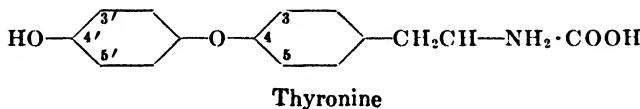
content of the cell increases (272) and there are characteristic changes in the nucleus and Golgi apparatus which will be described presently. If this maneuver does not suffice, the factory is enlarged further by hyperplasia of the

parenchymal layer of thyroid cells. Under pathological conditions enormous cysts may be formed by repetition of this process.

It is still uncertain how the colloid reaches either the follicle or the bloodstream. Among others, Bensley (36) has postulated a reversal of "polarity" in the cell, so that at certain times the cells secrete inward into the follicle and at other times outward into the bloodstream. Much of the data bearing on this subject is based on the tacit assumption that the colloid *per se* is the true secretion. More recent chemical evidence, however, suggests that the colloid may be broken down into smaller molecules before it is released from the follicle (400). It seems undeniable, in any case, that under stimulation by the thyrotropic hormone, the position of the nucleus and of the Golgi apparatus (as well as the form of the latter) may change considerably (401) (see Fig. 4). More recent observations in the presence of goitrogenic agents like thiouracil, however, suggest that these phenomena indicate cellular activity but not necessarily secretory accomplishment, i.e., the gland may show these physiological evidences of supreme effort while the parent organism sinks steadily into myxedema.

## II. The Thyroid Hormone

It is convenient to use the phrase "thyroid hormone" as a generic term to indicate any substance that will relieve human myxedema when properly administered. This definition implies that the hormone may occur in several forms, but that these must have a common denominator which is the chemical mediator of the specific effect. These materials vary in their physical and chemical constitution, but they all contain iodine substituted in the inner aromatic ring of a chemical grouping named "thyronine" (178). The thyronine framework consists of a diphenyl ether combination flanked at one extremity by a phenolic hydroxyl group and at the other extremity by a substituted alanine chain. Thus the hormone is at once a phenol and an amino acid.



The full name of thyronine is 4-(4'-hydroxyphenoxy)-phenylalanine.

Later it will be shown that certain exceptions to the above statement must be made. For example, low thyroid-like activity may be attained with bromine compounds.

### A. VARIOUS FORMS OF THYROID HORMONE

It is not yet generally appreciated that the term "thyroid hormone" no longer has a discrete chemical significance. Rather it is a biological word

indicating a substance whose administration causes a specific sequence of physiological events. Outstanding among the substances which produce the characteristic effects are the following:

- 1) thyroglobulin, the characteristic protein present in the "colloid" of the thyroid follicle;
- 2) its various degradation products, sometimes referred to as albumoses, peptones and polypeptides;
- 3) thyroxine (229) derived by the drastic hydrolysis of the foregoing or synthesized chemically (181);
- 4) other derivatives of thyronine; notably diiodothyronine, which is a simplified thyroxine derivative containing only two atoms of iodine per molecule instead of four;
- 5) probably sundry derivatives, as yet unidentified, of albuminous material resulting from the iodination of protein with subsequent hydrolysis and other isolation procedures. Salter (424) has described some of these in crystalline form isolated in small amounts from hydrolyzates of iodinated protein. They are presumably derivatives or congeners of thyroxine. These various substances all exert a characteristic biological effect and (with minor reservations) produce the complete effect of the natural thyroid hormone.
- 6) For the sake of pharmaceutical completeness, this list should include the whole thyroid gland. This usually is administered in the form of a dry powder from which most of the fat has been removed by acetone extraction in order to avoid unpleasant decomposition products.

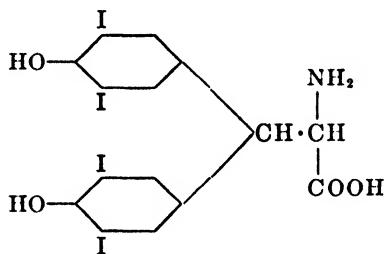
There should be added to these categories two other forms which are not frequently mentioned:

- 7) The circulating hormone. Obviously this substance transports through the blood stream the specific effect of the endocrine material elaborated by the gland. Present evidence indicates that this circulating hormone may not be an entity, but is actually built into the circulating plasma protein in much the way that antibodies are built into the circulating gamma globulin. One should not speak of "blood thyroxine" except in unusual chemical degradations.
- 8) Finally, there is that iodine-containing moiety which ultimately penetrates within the cell and produces the characteristic effects on metabolism and metamorphosis. Just how this end-organ form of the hormone integrates itself with cell mechanisms, and particularly with intracellular enzyme systems, still remains a matter of conjecture. It is identified in crude fashion by the presence of organic iodine within tissues. Whether it occurs in the cell sap is not yet proved. Possibly some day a particular intracellular "thyroenzyme" will be isolated and then, perhaps, we shall gain insight into the mechanism by which it exerts its final effect. Salter and Johnston (420,421) have prepared crude protein fractions from skeletal

muscle and have shown that the organically bound iodine is not uniformly distributed among the albuminous constituents of muscle, and does *not* behave like thyroxine.

Therefore, in speaking of the "thyroid hormone" it must be borne in mind that this generic term does not specifically imply the pure crystalline amino acid which was named by Kendall (230) "thyroxine." It is obvious, of course, that all of these materials comprise a single common denominator, and various studies have been made to identify this chemical *sine qua non*.

Some of the earliest studies of this nature were made by Abderhalden and his associates (3), and it was soon established that a high content of iodine was essential. The effect of the hormone is due, in addition to iodine, to the configuration named by Harington as "thyronine." The high specificity of this combination was demonstrated (182) through the synthesis of an isomer called "iso-thyroxine", i.e.,  $\beta,\beta$ -di (3,5-diido-4-hydroxyphenyl)- $\alpha$ -aminopropionic acid.



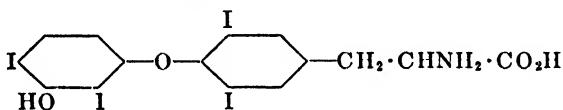
The proximate analysis and molecular weight of this substance are identical with those for thyroxine. This substance proved to be inert, thus showing that the thyronine configuration is highly essential.

When the two outer iodine atoms (3',5') of thyroxine were removed, the material lost over 90% of its activity. Nevertheless, this diiodothyronine, having only 4% of the activity of thyroxine on human myxedematous patients (14), qualitatively produced all of the features of the original thyroxine or of whole thyroid substance. Therefore, one must conclude in pharmacological terms that diiodothyronine is the basic hormone or "pharmacogen." The 3',5' iodine atoms, accordingly, would be considered as "auxopharmacophore" groups, namely, groups which enhance the effect of the "pharmacogen." When all four iodine atoms are removed the substance has no discernible activity, and when they are replaced by bromine, chlorine or fluorine (2,336) or various combinations thereof, very little activity remains. The tetrabromo-compound, however, in high dosage or concentration will induce metamorphosis in tadpoles and will relieve human myxedema (383).

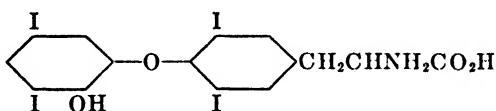
Various chemical modifications of iodothyronine have been made by Harington and his associates (182), Block and Powell (40), and Niemann and Mead (337), to explore the possibility that the thyroxine radical becomes modified in its native environment; i.e., that thyroxine is an artifact. The outcome of these laborious investigations is that the diphenyl ether configuration (181) and the two iodine atoms on the inner aromatic ring are essential. In very high dosage, to be sure, it is possible to produce weak thyroid effects from a variety of derivatives of thyroxine. For example, Woolley (536) prepared several ethers of *N*-acetyldiiodotyrosine. These not only antagonized the action of thyroxine, but the nitro ethers showed a weak thyroidal activity, in addition to their antithyroxine action, both in tadpoles and mice. Of these peripheral blocking agents the following order indicates decreasing effectiveness: para-nitrophenylethyl ether, paranitrobenzyl ether, benzyl ether and butyl ether. No blocking action was demonstrated with *N*-acetyldiiodotyrosine and *O*-methyl-*N*-acetylthyroxine. In similar fashion Frieden and Winzler (138a,532a) studied the effect of peripheral blocking agents on amphibian metamorphosis and as antagonists to thiouracil in rats. They found a weak but unmistakable activity in 3,5-diido-L-tyrosine, in *N*-acetylthyroxine, and in a glycine homologue and a benzoic acid analogue of thyroxine. Moreover, the two compounds last named showed a definite thyroxine-like effect. These findings suggest that, in addition to a side-chain containing some functional group and a hydroxyl group ortho or para to the ether oxygen, the essential chemical structure of the hormone itself is the orthodihalogenophenolic diphenyl ether configuration. They conclude that the side-chain in thyroxine is not highly specific. Likewise, Cortell (78) studied five analogues of thyroxine by noting their ability to prevent hypertrophy of the thyroid in rats treated with thiouracil. In a dose 150 times that of thyroxine (for marked activity), no activity was found for 3'-fluoro-DL-thyronine and only slight activity for 3'5'-diido-4-(4'-hydroxyphenoxy)-3,5-diiodohippuric acid. On the other hand, 3'-fluoro-5'-iodo-3,5-diido-DL-thyronine was at least one-third as active as thyroxine. Similarly, 3'-fluoro-3,5-diido-DL-thyronine proved to be about 1/30 as active as thyroxine and 3',5'-difluoro-3,5-diido-DL-thyronine about 1/50. None of these five compounds antagonized thyroxine in a dose 150 fold that of the accompanying thyroxine. Such observations as these indicate that the earlier concepts of the specificity of the thyroxine molecule must be modified if very weak activity is to be considered as significant. Rawson (373) has shown that in high dosage tetrabromothyronine will relieve human myxedema and so will the diphenyl thioether analogue of thyroxine. For practical purposes, however, the diiodothyronine framework is of the essence, and the presence of iodine in the molecule in strategic positions as cited greatly increases the activity. If one is willing

to consider substances with weak thyroid-like action, the specificity just described becomes less rigorous. Lerman, Richards, Brady and Riggs have found in human myxedema that seventeen molecules of tetrabromothyronine are equivalent to one molecule of thyroxine. Likewise 250 molecules of tetrachlorothyronine are required per molecule of thyroxine. On the other hand Cortell has shown that 2',6'-diiodothyronine exhibits no thyroxine-like activity, even though it is effective as a peripheral thyroxine-blocking agent. These data were shown to the American Goiter Society, Madison, 1949.

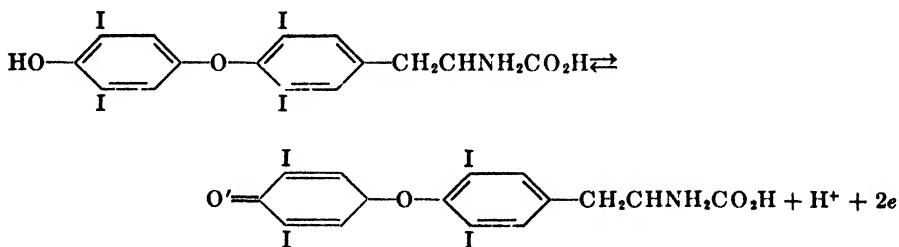
A few examples will illustrate this point. The precise location of the substituted iodine atoms in the thyronine configuration is critical. If these iodine atoms are misplaced in the molecule, a marked loss of biological potency occurs. For instance, when the 3',5' iodine atoms are transposed to the 4',6' position, the resulting compound becomes essentially inert.



Of course, during this transposition the 4' phenolic hydroxyl group is transposed to the 5' position. This loss of activity led Niemann (338) to postulate a quinoid form of thyroxine which might normally be the activated member of an oxidation-reduction system. Partial confirmation of the quinoid theory was obtained by the finding (337) of 4% activity in an isomer of thyroxine, i.e., **DL-3,5-diido-4-(3',5'-diido-2'-hydroxy)-phenylalanine**.

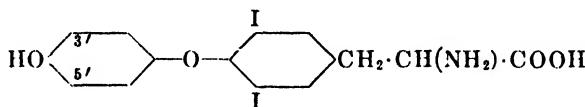


This quinoid formation could perhaps explain the remarkable effect which the hormone has on biological oxidation. According to this new hypothesis, an equilibrium would exist between thyroxine and the quinoid form:

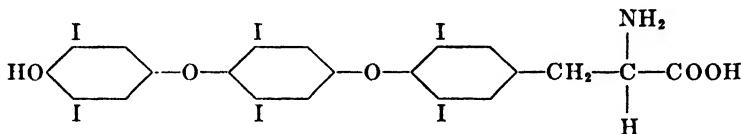


Any derivative of thyroxine which was unable to undergo quinoid transformation would be inert biologically.

This theory is interesting, but the evidence is not convincing. True, the omission of the 3',5' iodine atoms to form diiodothyronine



does not totally destroy activity, although only 4% of the original potency remains (259). When, however, a three-ringed amino acid is produced (51), as indicated by the following formula,



no significant biological activity is left. Moreover, the inertness of Harington's (182) "iso-thyroxine", already described, indicates the necessity of a diphenyl ether configuration. In short, as yet there is no clear picture of the chemical mechanism through which the characteristic effects of thyroxine are produced.

The problem is somewhat confused by studies of the metamorphosis of amphibian larvae, notably tadpoles. This type of test is much less specific than a therapeutic trial in human myxedema. Although difluorotyrosine is inert (264), the dibromo compound *does* stimulate metamorphosis and the gaseous metabolism of axolotl in large doses (1). Furthermore, diiodotyrosine itself causes tadpoles to undergo slow metamorphosis, although its effect is only 4% of that of thyroxine. Perhaps these halogenated tyrosine derivatives might be hormone precursors. Nevertheless, even potassium iodide, in tenfold the concentration required of thyroxine, will cause tadpoles to metamorphose prematurely (540), so that such amphibian experiments are of questionable value for investigators primarily interested in mammalian metabolism.

#### B. PRODUCTION AND SUPPLY OF THYROID HORMONE

It is perhaps an academic question whether thyroxine should be considered the ultimate tissue hormone, or merely its prosthetic group. One of the physiological observations which emphasizes this question is the characteristic lag in the response of mammalian metabolism after the intravenous injection of thyroxine. The suggestion has been made that some chemical change must occur before the material is able to participate in natural biochemical systems (421). As regards the optical activity of the prosthetic

group, Harington and Salter (186) showed that natural thyroxine is levorotatory, and that it exists in peptide linkage within the thyroid gland as an integral part of the protein, thyroglobulin. More recently, using the carefully fractionated human plasma of E. J. Cohn (73), Salter and his associates (31) have shown that the iodine is organically bound in the plasma protein and is largely concentrated in the crude albumin fraction when first fractionated. On further purification there is a sharp peak in the alpha-beta globulin fraction. Of course, both the plasma protein and the thyroid colloid contain an additional iodine-containing residue which can be isolated in the form of diiodotyrosine. Indeed, there is also evidence (428) (61) that muscle and ovarian tissue contain analogous "D" and "T" fractions. In short, wherever we are able to study the thyroid hormone derived from a natural environment, we find that before drastic degradation of the material has occurred, *e.g.*, by alkaline hydrolysis at high temperature, the physiologically active entity is bound to some albuminous substance (404). Recent experiments of Leblond and Gross suggest that the "D" fraction may also include monoiodotyrosine. Canadian Physiological Society, 1949.

Very recently, with the help of C. K. Drinker (110), Salter and his associates (427) (415) have been able to study the organically bound iodine of lymph. For this purpose they used a micromethod (426) which is extremely sensitive because it is based upon a catalytic reaction. By this technique, it is possible to work with 1 ml. of body fluid. It turns out that the protein-bound iodine of lymph and of pericardial fluid is less than that of plasma. The amount present could be predicted approximately on the basis that it escapes from the blood plasma bound to the albumin fraction of the plasma protein. Therefore, in lymph the ratio of bound iodine to protein is nearly twice that of plasma, which can be considered as "adulterated" by iodine-poor globulin.

In this way one can visualize the sequence of events through which the thyroid hormone passes. First, it escapes from the thyroid follicle through proteolytic action as shown by De Robertis (400). The polypeptide molecules are readily diffusible and can migrate into the blood stream via the rich network of capillaries which surrounds the follicles. Soon after this fraction arrives in the blood stream, it is incorporated into the circulating plasma protein and is maintained therein at a characteristic level. From this steady concentration of circulating hormone, a certain amount of the albumin escapes through the capillary walls into the interstitial fluid, bearing with it the characteristic constituent thyroxine which is now a part of the albuminous peptide chain (415). From this point, the path across the cell membrane into the cell sap is not understood. We know, however, that organically bound iodine exists in muscle (306) and is maintained at a fairly characteristic concentration unless the thyroid is removed. In that case,

TABLE I  
IODINE ( $\mu\text{g.}$ ) IN PERIPHERAL TISSUES\*

	Organ	Muscle	Protein fractions						Liver	Kidney	Heart	Serum				
			A			B										
			Fresh tissue $\mu\text{g.}/\text{g.}$	I/100 Total I g.	I/100 Total I g.	B	I/100 Total I g.	C								
Normal rats (20)		6.2 ± 0.9							5.2 ± 1.2	5.2 ± 0.7	6.2 ± 0.9	3.7 ± 0.7				
Treated with thiouracil																
1.00 g. daily for 10 days		5.0														
1.00 g. daily for 28 days		4.9														
0.25 g. daily for 90 days		3.2														
0.25 g. daily for 105 days		1.8														
Treated with thyroxine																
0.25 mg. daily for 5 days		8.2														
0.25 mg. daily for 11 days		20.0														
0.50 mg. daily for 10 days		21.0														
Normal cats (6)		25.0 ± 6.2														
Partition by ammonium sulfate			22.2	4.2	73.0	1.0	5.1	0.1								
Per cent partition			66	9.7	16	—	2	—								
Partition by acetone			98.7	252.0	1.0	—	—	—								
Per cent partition			85	85	8	—	—	—								
Human subjects (2)		5.3 ± 1.0														
Partition by acetone			14.8	0.27	19.5	0.17	64	2.13	5.5	6.4	—	6.8				
Per cent partition			11	7	7	82	82	2.6	3.0	—	—	2.8				

\* From Salter and Johnston, *J. Clin. Endocrinol.*, **8**, 924 (1948).

the tissue organic iodine is dissipated slowly and excreted as iodide. Salter and Johnston (420) have studied the protein-bound iodine in various organs. As shown in Table I, it all but vanishes in hypothyroidism induced by prolonged treatment with thiouracil. Contrariwise it increases to severalfold the normal value in hyperthyroidism induced by repeated injections of thyroxine. In its solubilities, it does not behave like pure thyroxine added to tissue extracts. When the muscle protein fractions (myosin and actin) described by Szent-Györgyi (490) are precipitated seriatim, the distribution of the protein-bound iodine is not uniform (Table I). Many years ago Kommerell (238) tested the specific dynamic action induced by feeding flesh from normal and hypothyroid dogs. The latter failed to increase the metabolism of recipient animals indirectly; and Kommerell concluded that it lacked a substance of thyroid origin. In general, the normal concentration of this tissue protein-bound iodine seems similar to that of normal human serum.

There are two possible sources of the thyroid hormone for general tissues: the endogenous material and the artificial supply of exogenous hormone which may be supplied to the patient. The thyroid gland itself tends to act, in fact, both as a factory and as a storehouse. Through this dual capacity it can regulate readily the supply of available hormone. The regulation depends largely upon the supervisory function of the pituitary gland which constantly secretes thyrotropic hormone and so maintains the gland in an appropriate state of activity. More will be said later of this regulatory action in the section on "The Pituitary-Thyroid Axis." There is another possible source of the hormone, namely in tissues outside of the thyroid gland. Asher Chapman (69) has shown that the salt and water balance in athyreotic rats deprived of iodine could be altered by increasing their iodine intake. The effect was similar to that produced by small doses of thyroid gland or thyroxine. By an entirely different technique Schachner, Franklin and Chai-koff (435) demonstrated that slices of intestine suspended in mammalian Ringer's solution could abstract radio-active iodine from the surrounding fluid and fix it in organic form. This finding has been interpreted as indicating an atavistic remnant of iodinating function present in all protoplasm, but other possible mechanisms merit consideration, e.g., atomic interchange. As far as athyreotic men are concerned, their extrathyroidal tissue activity does not protect them from myxedema.

Thyroglobulin was described many years ago by Hutchison (218) and Oswald (347) and is the storage form of the thyroid hormone. It is a very large colloidal molecule with an isoelectric point close to pH 5, coagulable by heat, and more soluble in salt solution than in water. One of the best preparations ever secured had a molecular weight close to 700,000 (195,196, 197). Later it was found that, in the presence of urea, this huge protein aggregate was split into four parts. It is not known whether the natural

aggregate is the quadruple form or the simpler molecule. However this may be, the protein is very large, comparable to the gamma globulin in blood plasma, and for this reason might readily be confined behind cell membranes or blood vessel walls.

Presumably the large size maintains the protein *in situ* within the thyroid follicle. Rienhoff (392) and De Robertis (399) have obtained samples of this material through tiny micropipettes inserted directly into individual follicles. The protein has been extracted by Cavett (63) and others from thick slices of thyroid tissue and has been analyzed for constituent amino acids by several workers (Table II A, B). Interestingly enough, it contains a

TABLE IIA  
THYROXINE AND DIIODOTYROSINE CONTENT OF THYROID PREPARATIONS\*

Preparation No.	Method of analysis	Total iodine %	"Extra tyrosine" %	Thyroxine			Diiodotyrosine		
				%	As iodine %	Per cent total iodine	%	As iodine %	Per cent total iodine
1	Brand and Kassell	0.425	0.23	0.25	0.17	39	0.44	0.26	61
	Leland and Foster	—	—	0.19	0.12	29	—	—	—
	Harington	—	—	0.33	0.21	50	—	—	—
2	Brand and Kassell	0.36	0.19	0.24	0.16	44	0.34	0.20	56
	Leland and Foster	—	—	0.15	0.10	27	—	—	—
3	Brand and Kassell	0.142	0.09	0.03	0.02	16	0.20	0.12	84
	Leland and Foster	—	—	0.01	0.01	5.4	—	—	—

\* From Brand and Kassell, *J. Biol. Chem.* 131, 500 (1939).

rather constant percentage of tyrosine congeners; namely, about 3.3%, which can be subdivided into three substituent portions, i.e., tyrosine itself, diiodotyrosine and thyroxine. In glands from colloid goiter which are poor in iodine, more tyrosine is found and less diiodotyrosine. In general, only about one-tenth is present in the form of thyroxine. In the average sample of normal thyroglobulin, therefore, it turns out that there are about six molecules of diiodotyrosine to one of thyroxine. The native protein must, therefore, be regarded simply as a storage form. From natural thyroglobulin, these two chief amino acids have been isolated in pure form. The first of these was known originally as iodogorgonic acid (103) because it was found in the coral *Gorgonia*. Later the substance was identified by Wheeler and Jamieson (525) as iodinated tyrosine, and Harington and

Randall (184) showed that it was a constituent of thyroglobulin. Using enzymatic digests provided by Salter (186) which had yielded L-thyroxine, these investigators (185) were able to isolate crystalline 3,5-diiodo-L-tyrosine. The isolation of these two amino acids by means of enzymic hydrolysis showed that these substances occurred in the levorotatory form and that they were bound in peptide linkage as an inherent part of the

TABLE IIB

## MOLECULAR WEIGHT OF THYROGLOBULIN FROM AMINO ACIDS AND COMBINING WEIGHTS\*

Constituent	Per cent found	Residues			Per cent calculated for different molecular weights			
		Number $N_t$		Fraction $F_t$	Total $N_t$	650,000	665,000	675,000
		Found	Cor-rected			650,000	665,000	675,000
Cystine (1)	4.30	238	240	1/24	5760	4.43	4.33	4.27
Methionine	1.31	59	60	1/96	5760	1.38	1.34	1.32
Total protein sulfur	1.46	303	300			1.48	1.45	1.42
Tryptophane	1.88	61	60	1/96	5760	1.88	1.84	1.81
Tyrosine	3.00	110	110	1/52.3	5756	3.07	2.99	2.95
Diiodotyrosine	0.64	9.8	10	1/576	5760			
Tyr. + diiodo	3.27	120	120	1/48	5760			
Thyroxine	0.32	2.7	2 or 3			Sum of deviations of the above found values from calculated		
Total iodine	0.58	30.4	(28 or 32)			0.29	0.12	0.20
Glucosamine	2.2	81.7	80	1/72	5760			
Carbohydrate	3.45	127	120	1/48	5760			

\* From E. Brand, B. Kassell, and M. Heidelberger, *J. Biol. Chem.* **128**, xi (1939) (Scientific Proc.).

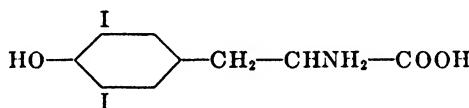
polypeptide chain of the protein. In short, diiodotyrosine and thyroxine do not exist as free substances in the thyroid in any significant concentration.

The natural thyroxine isolated by Kendall (230) was obtained from mammalian thyroid tissue after drastic alkaline hydrolysis. Its proximate formula is  $C_{16}H_{11}O_4NI_4$ . It contains 1.8% nitrogen and 65.4% iodine. Its struc-

tural formula is given on page 194. Its formal chemical name is L- $\beta$ -3,5-diiodo-4-(3'5'-diiodo-4'-hydroxyphenoxy)-phenyl- $\alpha$ -aminopropionic acid. It is a colorless, odorless microcrystalline powder which on rapid heating decomposes above 230°C.

Both diiodotyrosine and thyroxine give chemical reactions characteristic of phenol and of tyrosine. In addition, there is a characteristic color reaction (234) which consists in a cherry red color formed with nitrous acid under suitable conditions. As ordinarily prepared, thyroxine is precipitated from mildly acidic alcohol and (when pure) crystallized in sheaves or rosettes of long thin needles. When prepared from natural thyroglobulin through enzymic hydrolysis it is levorotatory; but as prepared synthetically, or as isolated from the gland by drastic alkaline hydrolysis, the material shows no optical rotation because it has been converted to the racemic form.

The proximate formula of diiodotyrosine is C<sub>9</sub>H<sub>10</sub>O<sub>3</sub>NI<sub>2</sub>. It contains 3.2% nitrogen and 58.7% iodine. The naturally occurring form is levorotatory. It crystallizes in white needles, melting at 199°C. Its structural formula is as follows:



In addition, a closely related substance, monoiodotyrosine, C<sub>9</sub>H<sub>10</sub>O<sub>3</sub>NI · H<sub>2</sub>O has been described (348). It gives the characteristic color reaction of Kendall and Osterberg, (234) with nitrous acid. It is not yet clear to what extent this substance exists in the thyroid tissue. Indeed, some authorities have questioned its existence and further study should be given it. Leblond believes it also exists in hydrolyzed serum.

Recently methods have been found for iodinating tyrosine *in vitro* with the resulting production of artificial thyroxine. This material will be described in a following section, but it should be noted that from levorotatory tyrosine one can make levorotatory thyroxine and from dextrorotatory tyrosine dextrorotatory thyroxine (382).

### C. BIOSYNTHESIS OF THE THYROID HORMONE

The mechanism whereby the gland elaborates its specific hormone was for many years extremely puzzling, because the synthesis (181) consumed many weeks; whereas synthesis took place in the gland within a few hours. The problem is now capable of partial explanation and resolves itself into at least three main stages: the fixation of iodide by the gland, the conversion of inorganic iodine into organic, involving the formation of diiodotyrosine, and the transformation of diiodotyrosine into thyroxine.

The thyroid gland is a remarkable iodine trap. Marine (290) showed many years ago, for example, that under appropriate circumstances the thyroid of a dog could retain 18% of a rather large dose of iodide injected intravenously. This material eventually would be converted largely into active hormone. Early in the present century, many attempts were made to produce the hormone directly by iodination of proteins; it was found (42) that when proteins were treated with iodine for several hours in the presence of bicarbonate, iodine was incorporated into the protein molecule. Proteins

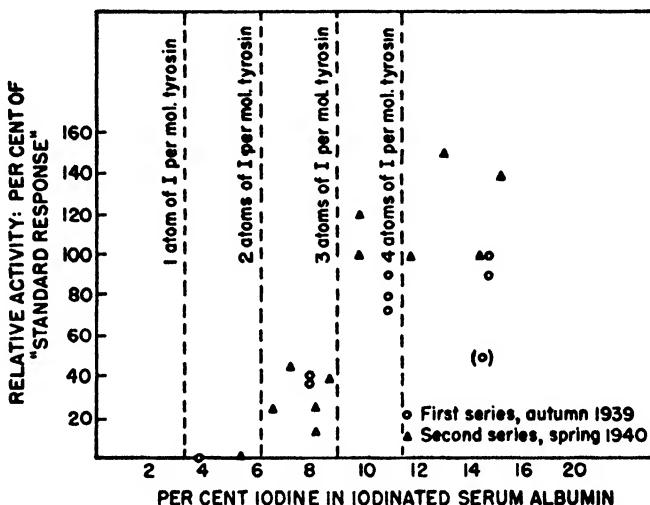


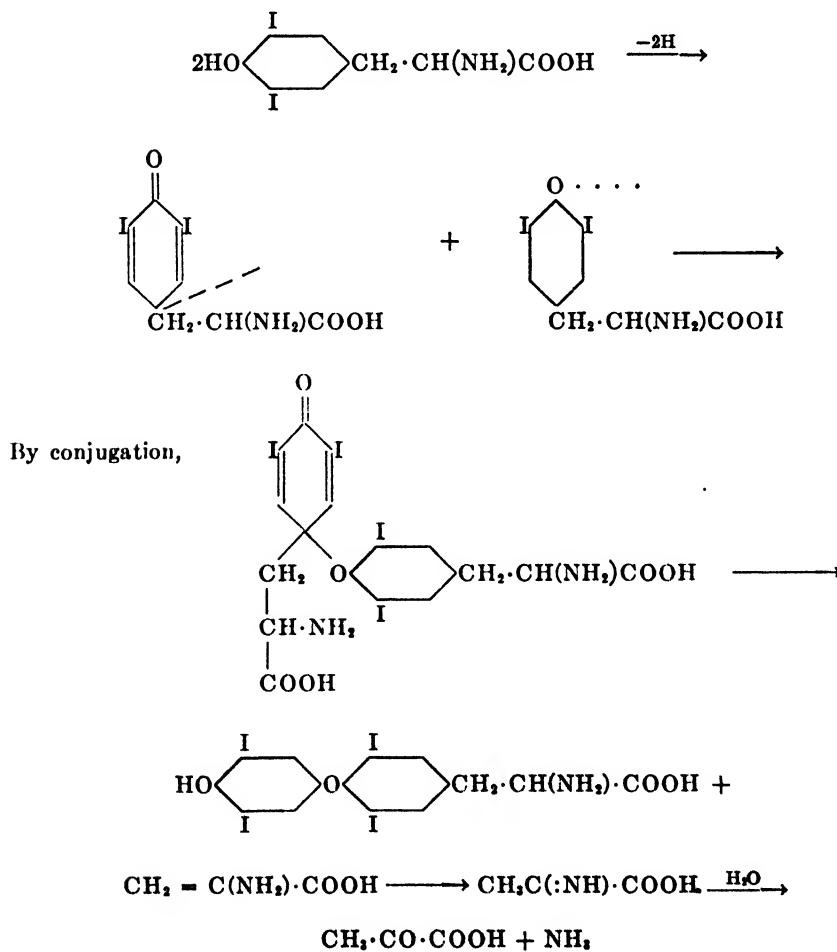
FIG. 5.—When serum albumin is iodinated, the constituent tyrosine must be converted to diiodotyrosine before thyroidal activity appears. (From Muus, Coons, and Salter, *J. Biol. Chem.* **139**, 141, 1941.)

as high as 7% in iodine content were obtained with egg albumen. The iodine is first incorporated into tyrosine radicals; later-reacting moieties of iodine participated in the oxidation of tryptophane groups, of cystine residues and of those groupings responsible for the biuret reaction. Until all of the tyrosine in serum albumin is iodinated, no thyroidal activity is produced (335); but thereafter one atom equivalent of iodine suffices to produce marked endocrine activity, as shown in Fig. 5. These findings, of course, suggested that an oxidative reaction was involved.

Turner and Reineke (384) have found that under appropriate circumstances as high as 4% yield of thyroxine can be produced by iodinating proteins. Thus excellent chemical data *in vitro* show that, by virtue of an oxidative procedure, tyrosine can be iodinated and then converted into thyroxine. Other proteins have been tried by Pitt Rivers and Randall (364). Salter studied gelatine, which is devoid of tyrosine, and found it use-

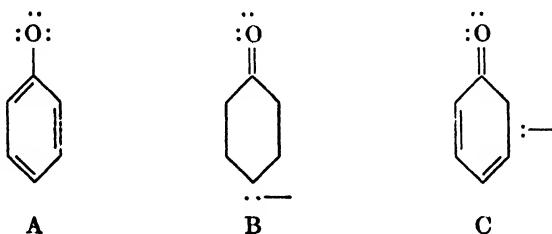
less. Dvoskin (113) has iodinated animal protein *in vivo* by injecting solutions containing elementary iodine into athyreotics suffering from myxedema. Their hypothyroid state was promptly relieved!

Following the lead of Abelin (4), von Mutzenbecher (334) showed that tyrosine itself could be iodinated in alkaline solution and would then form small amounts of thyroxine. Johnson and Tewkesbury (222) proposed a simple chemical oxidative mechanism as an hypothesis for the production of thyroxine. Based upon the studies of Pummerer (369), on the oxidation of *o*- and *p*-substituted phenols in alkaline solution, these workers studied the oxidation of 3,5-diiodotyrosine by hypoiodous acid (HOI). They postulated the formation of a quinolether intermediate as indicated in the following diagram:

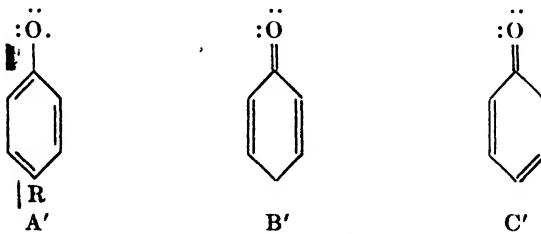


It will be noted that the end products of this series include ammonia and pyruvic acid. In point of fact, both ammonia and pyruvic acid were identified in the reaction mixture.

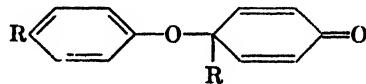
In Harington's laboratory (183) the experiments of Ludwig and von Mutzenbecher (278) have been repeated with various modifications. These results also confirm the findings of Reineke, Williamson and Turner (384) and of Block (40). There is no question that thyroxine can be obtained both by the iodination of proteins and by the treatment of diiodotyrosine with oxidizing agents in alkaline solution. The results of Block are considered particularly significant because the diiodotyrosine used was of synthetic origin. Harington (180) has extended the theoretical considerations of Pummerer (369) and of Westerfeld and Lowe (523) in terms of resonance. Three resonance forms of the ions are of special interest as follows:



Presumably the natural synthesis in the thyroid gland also involves such phenoxide ions, but under natural conditions these are substituted in the para position. Assuming that oxidation of these *p*-substituted phenoxide ions consists in the removal of one electron, the following three products would arise:



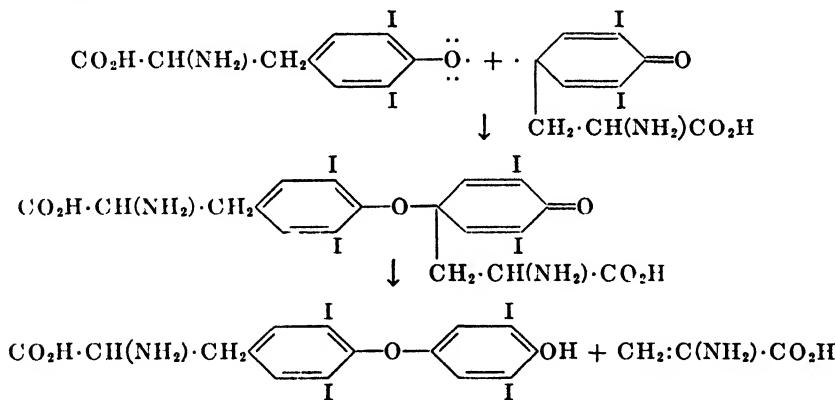
Self interaction of the last form can occur and would lead to the following compound:



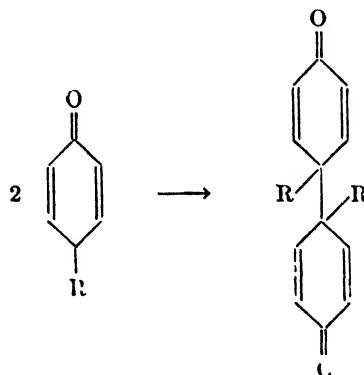
Such a compound has actually been obtained (369,523) for the case in which R = methyl.

Recently Harington (180) has developed conditions whereby diiodotyrosine can be oxidized directly to thyroxine by hydrogen peroxide in alkaline solution, and improved the yield through the use of a two-phase system in which the thyroxine is removed continuously by virtue of its preferential solubility in butyl alcohol. In this way he obtained a gross yield of 1.63% as compared with 0.23% obtained by von Mutzenbecher. The reaction proceeds best near pH 10, and while warm. These findings are confirmed by Turner (507) who found that the D-isomer had essentially no effect in combatting the goitrogenic action of thiouracil. Of special interest was his finding that from L-tyrosine the naturally occurring L-thyroxine was formed and that from D-tyrosine the rare (i.e., unnatural) D-thyroxine arose. During the process of oxidation a great mass of tarry by-products accumulates, just as when tyrosine is acted upon by tyrosinase in the formation of melanin.

Harington has suggested that the formation of thyroxine occurs by the following mechanism (cf. 523)



The theory is based upon the assumption that a compound of the following structure can react with itself as follows:



Thus the theory suggests a possible mechanism for the oxidative coupling of two molecules of diiodotyrosine to yield thyroxine. Moreover, it suggests that this reaction is rendered more likely by the presence of the iodine atoms in the diiodotyrosine.

Exactly how this reaction goes on in the thyroid cells is still a matter of conjecture. In surviving slices of thyroid tissue, iodine is fixed in organic combination by intact cells but not by homogenized cells (435). The reaction is inhibited in the complete absence of oxygen and by such substances as cyanide, azide, or hydrogen sulfide which inhibit cytochrome oxidase. It also is inhibited by such goitrogenic agents as thiocyanate and thiourea.

Salter (419) has suggested that in the intact gland the manufacture of thyroglobulin may occur independently of iodine metabolism. In other words, the crude globulin would constitute a biological scaffolding upon which free iodine or an equivalent iodase system might operate. Radioautographs by Hamilton (168) made from sections of colloid goiter after treatment with radioiodine, suggest that such iodine can be fixed rapidly by the stored abnormal thyroglobulin which was previously devoid of iodine. In studying this biosynthetic process the use of goitrogenic agents has been of special interest because it has been possible to produce blocks or "bottle-necks" at various stages of the gland's synthesis. Astwood and Bissell (21) showed that, after withdrawal of the drug, the injection of thyroxine or the removal of the pituitary retarded the accumulation of iodine and caused the acinae to become filled with colloid which contained very little iodine. In other words, protein synthesis proceeded without the formation of hormonal activity. Accordingly under the action of either thiocyanate or thiouracil, the organism ultimately suffers from lack of circulating thyroid hormone.

The most puzzling feature of the internal economy of the thyroid is the initial concentration of iodide. Ordinarily the fresh gland contains 0.04 % iodine. Of this, about one-tenth (or 4 mg. %) is inorganic. In other words, the concentration of iodide in the fresh thyroid is approximately 4000  $\mu\text{g. %}$  accumulated from surrounding fluids which contain only about one  $\mu\text{g. %}$ . How is this concentration gradient or differential accumulation of iodide maintained? The question could be answered with more assurance if the true concentration of free iodide were better known. The values in the literature vary considerably. Such technical factors as the deiodination of protein operate to make values too large, whereas the adsorption of iodide ions on protein precipitates tends to decrease the apparent inorganic fraction. Another possible source of confusion is the iodide which is loosely bound to the hypothetical enzyme system which regulates the iodination of tyrosine.

Recent evidence suggests that the so-called iodide of the thyroid can be subdivided into two categories; namely, the free and the bound  $I_F$  and  $I_B$  (419). As shown in Fig. 6 the free iodide is in equilibrium with the iodide of

the circulating plasma. In turn, fraction  $I_F$  is in equilibrium with the bound iodide,  $I_B$ , which is held in loose combination by a colloidal system which cannot pass cell membranes. This colloidal system is heat-destructible and, on coagulation, gives up its iodide readily. On precipitation with acetone, however, the iodide is precipitated in large measure with the protein. This colloidal system may be part of an enzyme complex, analogous to an oxidase system (416). The purpose of this "iodase" system would be twofold. The first component or "iodinase" could be supposed to combine with iodide and convert it into a compound analogous to a peroxide or hypoiodite. The second part of the system, the "periodase," would then conduct the oxidation of tyrosine to diiodotyrosine. It is also conceivable that one colloidal complex could subserve both functions. In any case it seems likely that some such enzymic system controls both the accumulation of inorganic

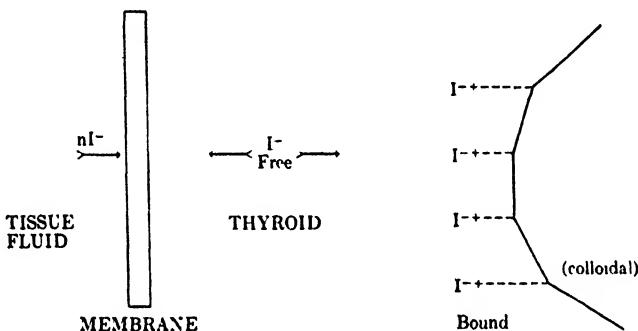


FIG. 6.—When free iodide ions enter the thyroid cell, some of these intracellular ions are loosely bound to a colloidal system. (From Salter, Cortell and McKay, *J. Pharmacol. Exptl. Therap.* **85**, 320, 1945.)

iodine and its subsequent incorporation into the tyrosine molecule. In this connection the model synthesis demonstrated by Keston (236) must be given great weight. This investigator employed xanthine oxidase, an enzyme found in milk and elsewhere, to transform iodide into the equivalent of elementary iodine. The end-product was diiodotyrosine. This enzymic synthesis of the natural precursor of thyroxine is undoubtedly an important clue to the mechanism whereby the thyroid gland manufactures its characteristic hormone from blood iodide.

It is likely also that some other enzyme system is concerned with the conversion of diiodotyrosine to thyroxine. The fundamental mechanism (179) has already been described. The finding (416) that under suitable conditions this stage is blocked by thiocyanate is further evidence that an enzyme system is concerned. Much more work must be done, however, before one can discuss these enzyme systems in precise terms.

### III. The Thyro-Pituitary Axis

Etymologically, pituitary experts are divided into two camps. The first group is interested in the pituitary secretion which *turns specifically* toward the thyroid, i.e., shows a *tropic* action. The second group is interested in the fact that the thyroid is *nurtured* or *maintained* by the *trophic* activity of the pituitary secretion. Many bitter words have been expended over that little letter "h." In the present section, the "h" is omitted because the context chiefly concerns the thyro-pituitary axis. In a later section, however, the "h" is included, because the subject matter chiefly involves the growth of thyroid parenchyma. No doubt both etymological schools will be displeased at this vacillation.

It is now well established that under ordinary circumstances the thyroid can exert only a minimal effort on its own initiative. This "resting state" can be altered through the specific stimulation by extracts of the anterior lobe of the pituitary. Schockaert and Foster (447) were among the first of many investigators who showed that extracts from the anterior lobe of the ox pituitary produced two major responses in ducks, guinea pigs, goldfish and several other species. These effects are:

(1) hypertrophy and subsequent hyperplasia of the thyroid gland, with resulting liberation of thyroxine-like material into the blood stream in increased amounts; and

(2) marked exophthalmos, often accompanied by general edema of the periorbital tissues, together with degeneration of the extraocular muscles. Brown Dobyns (94,95) has made one of the more recent studies of this feature of thyrotropic action. In guinea pigs, both intact and thyroidectomized, he produced a striking degree of exophthalmos by the daily injection of 25 Junkmann-Schoeller units of "antuitrin T" (Parke-Davis). Interestingly enough, a so-called purified thyrotropic preparation failed to produce significant exophthalmos, although it did cause hyperplasia of the epithelium of the thyroid (when present). Are there two distinct pituitary principles in ordinary preparations of this pituitary tropin? The "specific metabolic principle" of the pituitary was tested, but was found neither to stimulate the thyroid nor to produce exophthalmos. The eyes of intact animals, however, were less responsive than those of thyroidectomized guinea pigs. The converse experiment was also pursued with hyperthyroid patients subjected to subtotal thyroidectomy. The findings confirmed those of Soley (473,475), namely, that as the basal metabolic rate of some patients fell a correlated increase in exophthalmos occurred. Likewise thirteen patients afflicted with postoperative myxedema showed regression of the globe when treated with thyroid medication. It should be emphasized that these measurements were based upon true proptosis of the globe and not mere "stare". The pathological distinction between stare and true

exophthalmos is discussed elsewhere. As shown in Fig. 7, however, retraction of the upper eyelid due to increased activity of the sympathetic nervous system may produce an appearance opposite to that of Horner's syndrome. Nevertheless, this phenomenon is distinct from true exophthalmos.

#### A. THYROTROPIN AND THYROID FUNCTION

In the last century Nièpce noted that in a goitrous cretin the pituitary might attain a weight four times the normal. In this century Smith (469) and Allen (9) showed the converse phenomenon, namely, that the thyroid

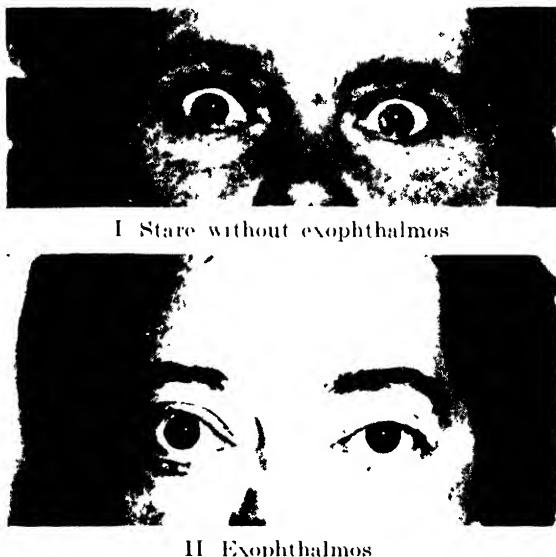


FIG. 7 It is important to distinguish between stare and true exophthalmos. (By courtesy of Doctor Mayo H. Soley. Cf. Salter and Soley, *Med. Clinics N. Amer.*, 1944, 490.)

became involuted after removal of the pituitary. This involution could be prevented or reversed by implanting tissue from the anterior pituitary, or by injecting crude extracts of that gland. Concomitant changes in oxygen consumption were also demonstrated (135). After removal of the hypophysis, the thyroid becomes smaller, but the iodine concentration increases. Probably the parenchyma shrinks while the deposits of colloid which are replete in iodine remain undisturbed. Therefore the total amount of iodine is decreased somewhat. Gradually the basal metabolism of the animal falls to some 80% of normal, but the organism stops short of definite myxedema. Apparently there is some natural activity on the part of the thyroid which does not require the so-called tonic stimulation of the pitui-

tary hormone, at least for a time. After a prolonged period, further atrophy of the thyroid may occur.

Just how the thyrotropic hormone exerts its activity is unknown. In addition to gross activation of the gland there are also evident changes in enzymic activity and distribution. In the activated gland peroxidase becomes demonstrable in the intrafollicular colloid as well as in cells (402). In resting (peripheral) follicles in the rat's gland the predominant phosphatase is the alkaline glycerophosphatase, whereas in active (central) follicles the acid phosphatase predominates (91). The activity of the cytochrome system is enhanced in stimulated glands, and this is inhibited by goitrogenic agents (434).

When the tropic hormone is injected, characteristic changes occur. If, for example, daily injections of a partially purified extract are made intraperitoneally in mammals, the follicle cells enlarge and continue to increase for several days. With larger doses, the initial hypertrophy is followed by hyperplasia, with active mitotic division. Simultaneously the margins of the plugs of colloid become scalloped and less eosinophilic. In the course of a few days the colloid gradually disappears and the follicular membrane becomes so hyperplastic and in-folded that it bears a close resemblance to the gland found in severe human Graves' disease. During this process, in spite of the loss of the thyroglobulin, the gland increases in size and weight for many days because of the increased vascularity and the larger mass of cytoplasm. If progressively increasing doses of the extract are used continuously for several weeks, cumulative effects occur (270) and at length the animal succumbs to the classical sequelae of hyperthyroidism. With smaller doses, however, (75,139) the animal may develop an apparent immunity to the extract made from tissue of a foreign species and become refractory to its physiologic influence (520).

Within recent years it has become clear that very early effects can be demonstrated in the thyroid after the parenteral administration of partially purified thyrotropic preparations. Thus, the water and chloride content of the guinea pig's follicular cells increase within 24 hours (272). In the meantime, the colloid stores of the gland decrease 50% and the oxygen consumption of the thyroid tissue increases 60%. Within a week the gland has increased to three times its original size. Also, the follicular contents exhibit an increased concentration of proteolytic enzyme (399). This effect may become demonstrable as early as 15 minutes after the injection of thyrotropic hormones.

If one studies further the iodine partition within the gland one finds that the loss of the thyroxine-like fraction occurs faster than that of the diiodotyrosine or of inorganic iodine (329). Presumably this reflects a dynamic equilibrium, in which the secretion of hormone is so speeded up that thyrox-

ine is released from the gland nearly as fast as it is produced. The accumulation of diiodotyrosine, however, reflects restorative processes, which tend to replace the waning stores with more material. Obviously the more recently accumulated colloid is likely to be less mature and to have a higher proportion of thyroxine-precursor in it. If thyrotropic stimulation persists, however, eventually practically no colloid can be demonstrated histologically and on analysis the gland is found to be nearly devoid of iodine. Such glands, if used as medication will have a little activity, but even less than would be anticipated from their meager total iodine content.

Obviously the results just described depend upon the supply of iodine. If this suddenly is increased the effect of the thyrotropic hormone may be nullified to a considerable extent in some species of animals, e.g., the guinea pig (139), the hypophysectomized rat, but *not* the chick (372). Precisely why this phenomenon occurs remains unknown, but it is obviously an important feature in the treatment of Graves' disease,—for instance, in preparation for operation. Apparently release of thyroglobulin from the gland is blocked, even though (under continuing thyrotropic stimulation) the synthesis of hormone proceeds apace.

The experiments of Rawson (226,246,247,380), have demonstrated two important features. The first of these is the capacity of explants of thyroid tissue to inactivate the thyrotropic hormone when bathed in a physiological solution containing an anterior pituitary extract. One normal rabbit thyroid will inactivate 10 Junkmann-Schoeller units. Explants of human thyroid tissue weighing 200 mg. have the capacity to inactivate 5 units; whereas an equal amount of tissue taken from a well iodinated gland of Graves' disease inactivates about 10 units. This inactivation is moderately specific because other tissues (excepting lymph node and thymus) do not affect the hormone's activity. It has also been observed that the gonadotropic hormone contained in the medium does not lose its activity. Dobyns and Rawson have also observed that the exophthalmos-producing factor of the pituitary is lost after exposing the hormone to slices of thyroid tissue. Thus under artificial circumstances it has been possible to mimic the reaction of the hormone with the gland as it presumably occurs *in vivo*. Indeed, thyrotropic hormone injected into a normal guinea pig cannot be recovered in any considerable amount (269,456); but when injected into the same animal after thyroidectomy, a high percentage of the parenterally administered material is recoverable in the blood or urine.

Secondly, Rawson and Albert (375) have demonstrated that the hormone which had been inactivated by exposure to slices of thyroid tissue can be reactivated by certain reducing agents, especially thiouracil and other goitrogenic agents. Since the reactivation in these studies has been produced by reducing agents, it is not surprising that in the test tube the hor-

mone has been inactivated by elemental iodine and that the same goitrogenic substances will reactivate the iodine-treated thyrotropic hormone. It is not proved, however, that the pituitary tropic hormone participates in the biosynthesis of the thyroid hormone, or even that it is necessarily concerned with the fixation of iodide by the gland. The various effects which thus far have been noted in the thyroid parenchyma as results of thyrotropic action can all be explained by the vague term "vital action". In short, the tropic hormone seems to activate cell mechanisms rather than participate directly in the formation of the thyroid hormone.

This is not to say that the tropic hormone does not influence the biosynthesis of the thyroid hormone or the fixation of iodine. After a single dose of tropic hormone, a precipitate loss of iodine occurs from the gland of the chick, reaching a minimum concentration in 24 to 48 hours (7). Thereafter, progressive storage of iodine occurs, so that by the ninety-sixth hour the stimulated gland has collected 300% as much iodine as the untreated gland. In short, the initial rapid loss of hormone is followed by a striking compensatory recovery (358,226). The interpretation of this "vital action" is still obscure. De Robertis (399) has suggested that the stimulated gland possesses an increased enzymic equipment wherewith to deal more effectively with its chief crude material, i.e., iodide. This increased enzymic activity involves proteolytic action both (1) in the stored colloid undergoing release and (2) in the cytoplasm of the follicular cells, which show alterations in mitochondria and in the Golgi apparatus (344).

The thyrotropic hormone is not effective by mouth, but in practically all species produces its characteristic effect when injected. Rather large amounts are required as shown by Schittenhelm and Eisler (440) and by Thompson (501), who produced hyperthyroidism in man within four days by the daily injection of 1000 Junkmann-Schoeller units of thyrotropic hormone. Unfortunately, within a few weeks or months species-specific antibodies develop and so check the action of the preparations available at present.

Recent observations involving certain sulfur-containing drugs which block thyroid function (20) have given an added impetus to investigations of the effect of the pituitary on the increased cellular activity of the thyroid (121,235,280).<sup>1</sup> Astwood and Bissell (21) have carried these observations further with thiouracil, as will be explained later. When examined by a trained pathologist, these thyroid tissues are practically indistinguishable from the primary hyperplasia found in severe human Graves' disease. Animals under the influence of these drugs are, however, suffering from a severe lack of thyroid hormone. This marked discrepancy between

<sup>1</sup> Austin Flint's textbook of Medicine in 1849 suggested the therapeutic trial of hydrocyanic acid in Graves' disease.

morphological appearance and actual function is important, because it demonstrates that the thyrotropic hormone from the pituitary can activate fundamental cellular reactions without necessarily participating in the synthesis of the thyroid hormone. In the intact organism these marked morphological changes are accompanied by functional pituitary activity. For example, in animals treated with thiouracil the pituitary shows changes characteristic of hypersecretion (419). Furthermore, if such an animal's pituitary gland be removed, further administration of the drug fails to change the appearance of the thyroid. Moreover, if in addition to the sulfur-containing drug an excess of thyroxine be given, the thyroid fails to show evidence of hyperactivity. One must conclude, accordingly, that within certain limits the activity of the thyroid is controlled by the pituitary and that this "thyro-pituitary axis" is influenced by the concentration of circulating thyroid hormone. It is well known that the response of the thyroid to exogenous thyrotropic hormone can be suppressed when thyroxine is administered simultaneously. When intact male rats are treated with thyroxine the mean cell height of the thyroid epithelium is depressed, but, if a sufficient excess of thyrotropic hormone be given in addition, the cell height can be raised to normal (79). In short, circulating thyroxine both suppresses the liberation of thyrotropic hormone by the pituitary and also diminishes the responsiveness of the thyroid tissue to the tropic hormone. Moreover, some sort of reciprocal quantitative relationship exists between the concentration of thyroxine and the effectiveness of the tropic hormone.

#### B. MODE OF THYROTROPIC ACTION

It is clear that hyperactivity in the thyroid ordinarily is the result of unusual thyrotropic secretion. Indeed glands perfused by a "Lindbergh heart" changed from a resting to a hyperplastic state as the result of the addition of thyrotropic extract (133). The effect produced could be essentially nullified by adding a high concentration of iodide. This antagonism to the reaction between the thyroid cell and the thyrotropic hormone has been substantiated by the observation that the inactivation of thyrotropic hormone *in vitro* by slices of thyroid tissue can be prevented if iodide is present in the medium (p. 311). Rawson and his colleagues (378) have shown that there is a peculiar and specific affinity of thyroid tissue for thyrotropic hormone (*cf.* also 269,456). Rawson's observations involved explants (in tissue culture) of normal and pathologic human thyroid tissues. Whereas explants from normal human thyroids inactivated a considerable portion of the pituitary hormone in the medium, explants of non-toxic goiterous tissue had no such effect. In contrast, slices of thyroid tissue removed from patients with Graves' disease inactivated twice as much pituitary hormone

as equal masses of normal human thyroid. Further studies (379) have employed cultures of rabbit thyroid grown in special roller bottles, coated with chicken plasma. When a pituitary extract was dissolved in the nutrient Tyrode's medium, it was found that this hormone was removed on exposure to normal thyroid tissue. When this tissue was later coagulated by heat, much of the pituitary hormone was released. One is reminded of the classical experiment which demonstrated the presence of pepsin in gastric juice through the use of cubes of beef! Besides thyroid tissue only lymph nodes and thymic tissue were found to remove thyrotropic activity from the Tyrode solution—and to a lesser extent. A variety of other human tissues failed to show any such effect. These results suggest that in thyrotoxic patients the hyperactive thyroid gland completely inactivates or removes thyrotropic hormone from the blood. Therefore none is found in the urine. (See also p. 311.)

It is far from clear what relation thyrotropic secretion has to exophthalmos. It is clear that an alkaline extract of the anterior pituitary can produce marked protrusion of the eyeball; and this effect can occur after most of the thyroid gland has been removed (292). The same result is found also after the removal of the upper cervical sympathetic trunk and ganglia. The mechanism, therefore, appears to be a direct chemical stimulation similar to that of estrogen upon the uterine mucosa. It may be assumed tentatively that the eye becomes involved whenever the thyroid fails to remove all of the tropic hormone from the bloodstream. It follows, if this assumption is correct, that after the surgeon removes a considerable proportion of thyroid tissue, the eyes may then become more prominent, because pituitary secretion circulates at an increased concentration. By actual measurement, this ocular protrusion occurs in about 40% of post-operative cases (94). This is to be regarded as another example of the so-called "castration phenomenon." In other words, according to this theory, ordinarily the thyroid gland partially protects the eye from thyrotropic hormone by specifically abstracting it from the bloodstream.

It may be assumed, further, that something analogous to a competitive affinity for thyrotropic hormone exists between the orbital contents and the thyroid gland. In exophthalmic goiter, both loci share the increased output of pituitary secretion. Part of the orbital change is due to edema (463) (5). The early experimental work of Loeb and Friedman (267) who produced exophthalmos in guinea pigs by injecting them with pituitary extract was extended by Pochin (369), who found definite exophthalmos, equal in normal and thyroidectomized animals, within 24 hours after subcutaneous injection. Unlike the chronic exophthalmos of human patients this experimental phenomenon can be explained quantitatively by edema of the orbital tissues, especially of the dorsal lachrymal gland and its sheath.

Perhaps the same is true of some of the more acute human cases. Aird (6), however, who also employed guinea pigs, was able to produce a persistent exophthalmos, with myopathy of extraocular muscles after prolonged treatment. These muscles showed the same edematous and hyaline degeneration (with lymphocytic infiltration) as in man. Apparently guinea pigs are less likely to develop the extensive increase in orbital fat found in human Graves' disease (407).

Various histological and chemical changes have been described in the extraocular muscles of exophthalmic guinea pigs and men. Smelser (464, 465) and Paulson (351, 352) described a Zenker's hyaline degeneration, accompanied by loss of striations, edema, and scattered foci of lymphocytes (*cf.* 6). There is indubitably an increase in the water content and volume of the orbital tissue mass. Such changes also affect the cardiac and other skeletal muscles of animals treated with large amounts of crude thyrotropic hormone. An edematous infiltration of the connective tissue of various fatty depots throughout the body occurs (466,96). The fat depots are depleted of lipoid, which is replaced by a more translucent gelatinous material. In the connective tissue edema occurs, with the infiltration of large numbers of polymorphonuclear leukocytes, lymphocytes and tissue macrophages. As the skeletal and cardiac muscle fibers lose their striations, they become filled with tiny fat globules aligned along the former cross-striations. At the same time, large amounts of fat appear in the liver, kidneys and epithelial cells, in the spleen, lymph nodes and large phagocytic cells of the lungs. These generalized changes occur regardless of the presence or absence of the thyroid. The plasma fat and blood acetone levels are elevated during these changes and there is a polymorphonuclear leukocytosis, characterized by fat-laden leukocytes similar to those found within the fat depots and connective tissue. Indeed, even the cytoplasm of the thyroid epithelial cells becomes "peppered" with tiny fat droplets during the administration of thyrotropic hormone. Likewise, Rundle and Pochin (407) have demonstrated an increase in the fat content of the eye muscles in exophthalmic goiter. Presumably a profound readjustment of fat metabolism occurs under these circumstances (15,96).

There is other evidence in favor of the hypothesis above. For example, in Graves' disease, immediately after subtotal thyroidectomy high concentrations of urinary thyrotropic hormone are found. Likewise, in the idiopathic cases of exophthalmos a high urinary excretion is often noticed, and likewise in myxedematous patients in whom the gland has atrophied spontaneously. To sum up, our present conception of exophthalmos in Graves' disease is that of a chemical effect of the pituitary secretion upon orbital tissues. If the thyroid sops up the pituitary substance responsible for the exophthalmos, then the eye will be protected at least partially.

This hypothesis must be substantiated by further work, but at least it serves as a tentative scheme for a logical discussion of thyro-pituitary mechanisms. Better means of measuring thyrotropic hormone are needed to test the theory.

If this hypothesis is true, then one must assume that the thyrotropic secretion tends to be checked by thyroxine. The evidence for this in animals is good (271) (17) (243) (266). Moreover, Salter and Soley (431) have described a diminution of idiopathic exophthalmos in human patients under high dosage of thyroxine. In this respect, it must be assumed that Graves' disease should tend to be self-limiting, because as the hyperthyroidism increases, a higher concentration of thyroxine will be applied to the hypothalamic center which controls the pituitary gland. Perhaps, too, thyroxine can act directly on the anterior lobe.

### C. GRAVES' DISEASE VERSUS HYPERTHYROIDISM

It was formerly believed that the cause of Graves' disease was purely thyroid hyperactivity. Another point of view, however, seems more reasonable: namely, that hyperthyroidism is *one* of the results of Graves' disease. One must recognize two extreme clinical states: the first is Graves' disease with normal or subnormal thyroid secretion. The other is Graves' disease with severe hyperfunction of the gland. This point of view was expounded by Falta (126) many years ago in Vienna. Among the actual population of thyroid patients, one finds all degrees of nervousness and exophthalmos with any degree of caloric turnover. The cases which create the greatest trouble in the clinic are those in which the metabolism is low and the exophthalmos pronounced.

### IV. The Thyro-Ovarian Axis

There are several mutual interrelationships between the pituitary on the one hand and the thyroid and ovary on the other. Formerly it was believed that there was a thyro-pituitary axis and an ovarian-pituitary axis and that all the mutual interrelationships were established exclusively through the pituitary gland. Recently, however, evidence has been adduced (511) which suggests that the thyroid also exerts an effect on the ovary directly; and the question has even been raised whether the ovary may have a direct effect upon the thyroid.

The characteristic enlargement of the thyroid during the latter part of the menstrual cycle and during pregnancy has been recognized since antiquity (333) and has even been incorporated into Medieval and Renaissance art. In the clinic it is still widely recognized that one of the most effective remedies in the armamentarium of the gynecologist is whole thyroid sub-

stance. For example, Haines and Mussey (165) in 1935 showed that in 50 cases of amenorrhea, catamenia was restored in 29 through the use of thyroid medication. Furthermore, in 9 cases of oligomenorrhea thyroid medication apparently restored normal menses in 3; and of 15 cases of menorrhagia 8 showed normal catamenia after thyroid medication.

In sterility and habitual abortion, also, the judicious use of thyroid is very important. For example, in 1914 Hertoghe (199) said that thyroid extract was an "excellent remedy for otherwise inexplicable sterility. Many women who have taken it for obesity have become pregnant," unexpectedly. Apparently thyroid medication augments or facilitates ovum formation. The best interpretation of the available data suggests that this is not a very specific effect, but rather is due to its action upon growth or differentiation of tissues in general. In this connection it will be remembered that in hypothyroidism, human fertility declines; and during early pregnancy, if hypothyroidism persists, the fetus is likely to die in utero. On the contrary, cases of habitual abortion may go through a normal pregnancy on about 2 grains of U.S.P. thyroid daily (281). In the bovine species, however, as illustrated in Fig. 8, this situation may not hold.

In pregnancy, of course, there is an increased requirement for thyroid hormone and a slight tendency for an increase in over-all basal metabolic rate. Boothby (49) found in 30 cases of pregnancy that 80% had normal basal metabolic rates; in 3 cases the rate was between plus 16 and plus 20, and in 3 others it exceeded plus 20. The thyroid, particularly in the first half of pregnancy, is doubtless supplying hormone for an increased mass of body tissue; in the last third of human pregnancy, the fetal thyroid can suffice for the fetus and even for the hypothyroid mother in a rare instance. It was thought formerly that pregnancy could not endure during marked hypothyroidism in women. The protein-bound iodine of the serum rises during pregnancy (361). In comparison with the normal concentration of from 4 to 8  $\mu\text{g. } \%$ , in 43 cases of early pregnancy the range rose to 6 to 10  $\mu\text{g. } \%$ . This slightly elevated concentration declined soon after delivery. In certain cases of early miscarriage the slight elevation of presumably "hormonal" iodine failed to occur. In such patients, the values lay between 2.8 and 5.8 and presented an indication for thyroid medication. On the other hand, in cases of infertility and in toxemia of pregnancy the circulating hormone was usually normal. It must not be inferred from these values that other species react in the same way. For example, hypothyroidism does not seriously influence the continuance of pregnancy in guinea pigs (539), and hypothyroid cattle reproduce successfully (353). Even in man occasionally pregnancy continues in the presence of a marked lack of thyroid hormone.



FIG. 8 A



FIG. 8 B.

FIG. 8.—In the bovine species hypothyroidism of considerable degree does not interfere with reproduction.

A. Thyroidectomized sire of the calf shown in B.

C. The dam in mid-pregnancy illustrating the characteristic puffiness of the hocks, the bloated rear quarters and poor mammary development.

D. The same dam immediately after parturition, illustrating the steady improvement in thyroid status which occurred in the latter half of pregnancy. Except for the rather small udder, the dam is not abnormal. (By courtesy of Professor W. E. Petersen, University of Minnesota, Department of Agriculture.)



FIG. 8 C.



FIG. 8 D.

There is probably some reciprocal relation between thyroid and ovary which is not well understood. Females, for example, are much more frequently affected than males with toxic goiter; in the proportion of approximately 4 to 1, although lower ratios apparently occur in endemic goiter regions. Moreover, once a nodular goiter has become toxic, or after frank exophthalmic goiter has become established in the course of pregnancy, the patient is very likely to abort. The history is often that of a "nervous breakdown" leading eventually to spontaneous interruption of pregnancy. In other cases the "spells" hurry the birth of the child and may occur repeatedly after an excessive number of pregnancies.

There is some evidence that the ovary participates directly in iodine metabolism. For example, in studying the "thyroid index" i.e., the ratio of thyroid weight to body weight, McCarrison (303) found throughout the entire life span of several species of animals that this index became maximal just before the beginning of sexual maturity. Moreover, the iodine content of the ovaries is higher than any tissue in the body except the thyroid (299). It is lower before puberty and after the menopause (300). Values in human tissue as high as 30 to 160  $\mu\text{g. \%}$  (486) are reflected also in other species: for example, in bitches 104 to 161  $\mu\text{g. \%}$ ; in swine 609 to 648; in guinea pigs 340; in rabbits likewise 340 (299). Carter (61) has also detected a cyclic change in the ovarian iodine in association with ovarian activity. New-born babies have a lower ovarian concentration (i.e., 138  $\mu\text{g. \%}$ ) than do adult women (741  $\mu\text{g. \%}$ ) (300). The presence of ovarian lipoids makes such analyses difficult, and more studies are needed using modern techniques. Perkin and Brown (355) have shown that in male dogs thyroideectomy alters considerably the response of the blood iodine to the administration of iodide. In the female, however, very little alteration occurs after thyroideectomy, but a quite pronounced change occurs when both ovary and thyroid are removed. Furthermore, there are minor fluctuations in blood iodine during menstruation and pregnancy which suggest that the thyro-ovarian axis has a concomitant effect upon thyroid activity (458).

It still is uncertain how effective the thyroid is in modifying the response of the peripheral tissue, e.g., the ovary, to gonadotropic hormone. Nevertheless, there seem to be a number of reciprocities between thyroid and ovary which suggest such peripheral action: for example, an increase in the milk production of cows (158,38) and in egg production of hens (529), after thyroid feeding. Membrives (322) studied in immature rats the ability of implants of the anterior pituitary to produce vaginal cornification through ovarian stimulation. He found that the effect was augmented by feeding thyroid and diminished by extirpation of the thyroid gland.

Tyndale and Levin (511) studied the ovarian weight of (a) normal rats,

(b) hypophysectomized rats, and (c) hypophysectomized immature rats treated with thyroxine. These animals were all injected with urine obtained from patients at the menopause, and therefore high in gonadotropic hormone. Ordinarily such injections produce considerable stimulation of the ovarian follicles, but the simultaneous injection of thyroxine (group c) decreased this response considerably. These results in hypophysectomized animals suggested that the inhibitory effect of the thyroid was a peripheral one, not involving the pituitary gland. Gessler (146) has also shown that estrone causes a decline in metabolic rate both of normal guinea pigs and of hypophysectomized rats. Another illustration of such synergistic action may be found in work on the response to growth hormone when potentiated by thyroid. Both hypophysectomized and hypophysectomized-thyroidectomized rats respond better to known doses of growth hormone when treated with thyroxine concomitantly. This fact was demonstrated by P. E. Smith (470) and confirmed by Evans, Simpson, and Pencharz (124) in studies of body weight response. The synergism between the thyroid and the pituitary in producing growth can also be demonstrated by the use of thyrotropic hormone in hypophysectomized rats. Stimulation of the animal's own thyroid also increases the response to growth hormone (295). Through the kindness of these authors, the following, hitherto unpublished data (462) may be cited. The growth hormone was a cysteine-treated globulin fraction; its potency (in units) was calculated by interpolation on curves for both the 10-day and 15-day test period as described by Marx, Simpson, and Evans (296). These animals were hypophysectomized at 26–28 days of age, and used for the test 10–12 days later. The findings are illustrated in Table III.

Although it has been questioned whether completely thyroidectomized rats can be stimulated to grow by the injection of pituitary growth hormone, the early work of Flower and Evans (131) with rats thyroidectomized after weaning showed that such injections could preserve the normal growth rate. Indeed rats thyroidectomized on the first day of life were capable of responding to growth hormone (452,409) much later.

It must be admitted that these reciprocal thyro-ovarian relationships are still so complicated that a precise summary of them is not easily possible. There seem to be three general types of interrelationships occurring: (1) a peripheral sensitization of the tissues by thyroid hormone to ovarian hormone, and the converse; (2) an effect of the thyroid on the pituitary-ovarian axis and the converse; and (3) an indirect effect upon other glands, such as the indirect effects through the adrenal cortex which can affect sex activity. Indeed Starr (480) attempted to control clinical hyperthyroidism by administering estrogens.

An example of antagonism between estrin and the pituitary-thyroid axis is found in the experiments of Cramer and Horning (84). They found that injections of thyrotropic hormone prevented the characteristic effects of the prolonged administration of estrin in mice, i.e. enlargement of the mammary gland in male mice, the spontaneous development of mammary carcinoma, and characteristic changes in the oxyphilic granules of the pituitary gland. On the other hand, Franck (134) found that the injection of huge doses of estrone into guinea pigs yielded "quiescent" thyroid glands.

Almost nothing is known about differential sensitivity to thyroid hormone in the peripheral tissues. In the clinic the most obvious manifestation of this difference is the occurrence of localized myxedema (13). This disturbance sometimes occurs in patients with exophthalmic ophthalmoplegia

TABLE III  
COMBINED EFFECT OF THYROXINE AND GROWTH HORMONE IN HYPOPHYSECTOMIZED RATS\*

Treatment	No. of rats	Daily dose	10 Day test		15 Day test	
			Body wt. gain	Growth hormone units	Body wt. gain	Growth hormone units
Growth hormone	6	100 μg.	13.0 g.	18	17.0 g.	13
Growth hormone + Thyroxine	5	100 + 75	15.3 +	29	22.0	24

\* For these hitherto unpublished data the author is indebted to Dr. Miriam E. Simpson. Cf. Marx, Simpson and Evans, *Endocrinology* 30, 1 (1942).

or in so-called "burned-out" Graves' disease. In both instances, the concentration of circulating thyroid hormone is likely to be normal.

The problem is complicated by the fact that prolonged hyperthyroidism leads to exhaustion of the enzymic components of tissues, particularly thiamine, pantothenic acid and pyridoxine (105, see below). If sufficient to precipitate a frank vitamin deficiency, this may interfere with the tissues' responsiveness to thyroid hormone. Since the thyroid hormone operates in the range of catalytic concentration found for the hydrogen ion, thiamine and digitoxin (415) it may act by becoming the prosthetic group of a "thyrenzyme" system (416). The effectiveness of such a "thyrenzyme" component (421) would be conditioned by the oxidation-reduction and hydrolytic mechanisms with which it interdigitates.

## V. The Circulating Hormone in Plasma, Lymph, and Other Body Fluids

In both health and disease a complicated series of equilibria intervene between the thyroid gland and the peripheral tissues. It is essential to bear this in mind in considering normal thyroid physiology and its perversions in pathological states. The gland manufactures and stores the hormone, so that inorganic iodine is converted, first, into diiodo tyrosine and, later, into thyroxine. Even if this step-wise synthesis occurs in the follicular cells, the resulting product is stored within the follicular spaces. From the gland the hormone is released under the influence of the thyrotropic action of an anterior pituitary hormone according to some bodily need or under perverted stimulation in the case of exophthalmic goiter. Probably the concentration of iodide in the plasma exerts a sort of back-pressure effect on such hormonal release: so that even during such a hyperactive stage there exists a complicated relationship between the gland and the blood plasma. Ultimately, however, under normal circumstances a dynamic equilibrium is established, such that a steady supply of hormone is taken up by the circulating plasma just equal to that which is removed by the peripheral tissues.

Thus a sort of homeostasis exists, in the sense of Cannon (57) with respect to the thyroid hormone. Whether this material is released as a thyroxine polypeptide is still unknown; but at any rate, very soon this internal secretion is incorporated in the blood plasma. For a few minutes, and possibly for an hour or two, after the administration of thyroxine intravenously, it is possible to demonstrate that the newly acquired thyroxine is not part and parcel of the plasma proteins. It is soon found, however, that the circulating hormone—now greatly increased above the normal level—is precipitable with the protein in the presence of organic solvents. These ordinarily would retain thyroxine in solution, separating it from the colloidal plasma proteins. For example, Salter and Johnston (420) compared the protein-bound iodine in three types of human serum, i.e., from spontaneous hyperthyroidism, from a normal individual, and from myxedema. The typical values found in these three physiological states were approximately 17, 5 and 2  $\mu\text{g. \%}$ , respectively. Then the latter two types of serum were re-enforced (i.e., "spiked") with additions of pure DL-thyroxine, so that all three samples of serum contained approximately the same concentrations of organically bound iodine. Thereupon all three were subjected to precipitation with acid acetone and the protein precipitates analyzed for iodine. As shown in Fig. 9, the added thyroxine in the latter two samples largely failed to be precipitated. On the contrary, the acetone precipitate from naturally "hyperthyroid" serum contained just as much protein-bound iodine as previously found by heat-coagulation. In short, naturally occurring organic iodine in serum behaves differently from pure thyroxine.

When the plasma protein fractions are studied carefully, two types of distinction can be made which throw considerable light upon the nature of the colloidal hormone as it is carried in the plasma. One such difference concerns the chemical nature of the iodine moieties in the plasma. Of course, the iodide is readily removed when the proteins are precipitated, but the protein-bound iodine, designated by "P," can be subdivided by hydrolysis into "D" and "T" fractions much like those found in thyroglobulin itself (31). In short, as the stores of hormone disappear from the thyroid gland they reappear in the plasma proteins. As shown in Fig. 10, the "D" fraction of plasma is about one-quarter to one-third of the total protein-bound iodine. In hyperthyroidism it increases further. Why there should be any

Acetone Precipitation of Serum  
Reenforced with Thyroxine

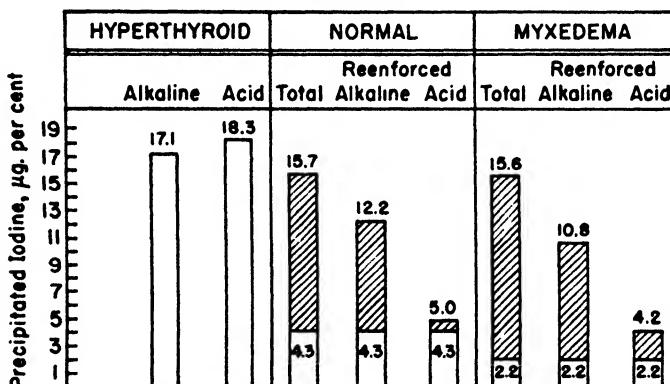


FIG. 9.—When serum from a normal or a myxedematous individual is artificially re-enforced with thyroxine, it does not behave like serum from a naturally hyperthyroid patient. (From Salter and Johnston, *J. Clin. Endocrinol.* 8, 920, 1948.)

"D" fraction in the circulating plasma remains unknown; because at the present date no known physiological function in the peripheral tissues can be assigned to diiodotyrosine *per se*. Experiments with radio-active iodine, usually with the isotope  $I^{131}$ , have been reported (161) which show that the plasma contains thyroxine. These observations indicate that the thyroidal activity conveyed by the serum resides in a prosthetic group which is probably thyroxine,—or possibly a very close derivative thereof. Many years ago Blum (41) discussed the possibility of an "iodase" in plasma which would favor the incorporation of iodide into the plasma proteins. Recently Swenson and Curtis (487) found that when iodide is added to fresh serum, the iodide ion is at first free. After hours and days of incubation, however, it appears to be bound to the protein. This phenomenon must be differentiated from the simple adsorption of iodide on plasma pro-

tein molecules, as described by Salter (411) in the presence of high concentrations of iodide. From a practical standpoint of diagnosis, both phenomena lead to "spurious" evidence of hyperthyroidism.

Before discussing the circulating hormone it is necessary, first, to consider the inorganic iodine of the body. This fraction confused the hormonal picture for several decades by presenting a technical adulteration of the true "hormonal" iodine. It was only when the separation of this iodide from the organically bound iodine became possible that a true picture of the hormone could be attained. In general, it would seem (516) that iodides are distributed in extracellular fluid like chlorides, as described by Peters (359). The iodide ion, like chloride, permeates practically all tissues, in-

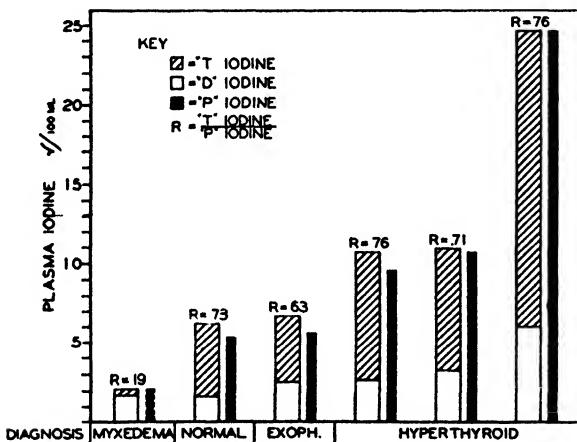


FIG. 10.—The circulating "hormonal" iodine in blood serum can be divided into "D" and "T" fractions. (From Bassett, Coons, and Salter, *Am. J. Med. Sci.* **202**, 524, 1941.)

cluding those transudates and cystic fluids which occur in pathological conditions. Characteristic values reported (516) are given in Table IV.

The actual concentration of circulating iodide varies with circumstances. In many regions, e.g., the Connecticut Valley or the goiter belt around Cleveland, the total iodide concentration is less than 1  $\mu\text{g. \%}$ . In these environments an individual may be in metabolic iodide balance with a urinary excretion between 20 and 200  $\mu\text{g. per day}$ . This "natural" level of metabolism, however, may be increased considerably if the physician supplies prophylactic medication to avoid simple goiter. This second or "prophylactic" level of metabolism may involve an iodine excretion of 200 to 1000  $\mu\text{g. per day}$ , and a plasma iodide concentration of 3 to 5  $\mu\text{g. \%}$ . Still higher plasma levels are achieved in the therapy of exophthalmic goiter which frequently produces an excretion between 2 and 75 mg. of

iodide a day. At this therapeutic level, the plasma total iodide may reach well over 60  $\mu\text{g.}$  % even 18 hours after the usual dose. Finally, at the so-called "fibrolytic" level of dosage, namely after several gm. a day, the plasma iodide level may attain values of over one or two mg. %.

As mentioned, iodide in the circulating plasma and lymph has been until recently, of interest chiefly because it presents a technical hazard in the

TABLE IV  
DISTRIBUTION OF IODIDE AND CHLORIDE IN VARIOUS ORGANS AND BODY FLUIDS\*

	Iodide per g. wet tissue	Chloride per g. wet tissue	Per cent interstitial fluid calculated from	
	mg.	mg.	Iodide	Chloride
Serum.. . . . .	0.184	3.82	—	—
Liver. . . . .	0.050	1.14	27	30
Muscle. . . . .	0.031	0.49	17	13
Skin. . . . .	0.142	2.40	77	63
Lung. . . . .	0.119	2.42	65	63
Kidney. . . . .	0.119	2.35	65	61
Stomach. . . . .	0.097	2.00	53	52
Pancreas. . . . .	0.064	1.45	35	38
Spleen. . . . .	0.064	1.54	35	40
Adrenals. . . . .	0.062	1.40	34	37
Per g. dried blood			Ratio of dried blood to serum	
Cat	{ Blood. . . . . Serum (per cc.). . . . .	4.26 0.978	19.5 4.47	4.3 4.3
Dog	{ Blood. . . . . Serum (per cc.). . . . .	0.790 0.199	14.4 3.86	3.9 3.7
Rabbit	{ Blood. . . . . Serum (per cc.). . . . .	3.96 0.877	15.0 3.60	4.5 4.2

\* From Wallace and Brodie, *J. Pharmacol. Exptl. Therap.*, 61, 407-408 (1937).

analysis of "hormonal" iodine. When the higher dosages of iodide are used, both the plasma and the red corpuscles, as well as the interstitial fluid, the lymph, the cerebrospinal fluid and the milk show a high concentration of iodide. When radio-opaque "skiodan" is injected intrathecally, the serum iodine subsequently reaches concentrations about one-thousand times the normal as shown in Table V. Recent studies (421) (227) indicate, however, that the apparently insignificant iodide in serum and body fluids must receive more attention. For example, in the turnover-rate of thyroid hor-

mone in normal rats the concentration of iodide played a determining role in the calculation of the amount of thyroxine metabolized each day (421). Moreover, in observations involving radioactive iodide, Keating and Albert (227) have described both a "renal clearance" and a "thyroid clearance" of the iodide from the blood.

As already mentioned, the plasma has long been known to contain iodide in amounts depending upon the environment, but frequently less than one  $\mu\text{g. \%}$  in conditions of health. The precise nature of the main moiety of the

TABLE V

IODINE CONTENT OF SERUM AND SPINAL FLUID AFTER THE INTRATHECAL ADMINISTRATION OF "SKIODAN" (SODIUM SALT OF MONO-IODO-METHANE-SULFONIC ACID)<sup>a,b</sup>

	Patient	Before injection	Shortly after	$\frac{1}{2}$ hr. after	1 hr. after	2 hr. after	3 hr. after	4 hr. after	5 hr. after	24 hr. after
Spinal fluids, mg. %	E. R.	0.0			5.02	3.91				
	Mc. M.	0.0			8.0	1.94				
	B.	0.0			4.45	7.21				
	E. E.	0.0			9.62	5.48				
	C. M.	0.0			5.36	12.16				
	A. G.	0.0			2.64	3.54	7.95	0.00	6.78	0.00
Sera, mg. %	C. W.	0.04	0.54	3.25	4.26	2.70				
	L. P.	0.28	0.33	—	1.51	1.61				
	M. L.	0.01	0.53	1.11	1.85	2.92				
	L. deF.	0.02	0.07	2.72	3.37	2.67				
	E. S.	0.006	0.04	1.95	—	2.26				
	J. C.	0.07	0.05	1.25	1.60	1.13	0.00	0.00	0.00	0.00

<sup>a</sup> Random cases.

<sup>b</sup> From Salter, Munro, and McKay. Unpublished data (Cf reference No. 416)

plasma iodine, however, is still under discussion. It was once thought that this circulating "organic" iodine might be either thyroglobulin or thyroxine itself; or possibly a peptide of thyroxine. Indeed, Man *et al.* (287) found that thyroxine added to serum was largely precipitated with the proteins. However, pure thyroxine added to plasma behaved like potassium iodide, in that it appeared in the supernatant fluid when shaken with organic solvents. On the contrary, the plasma from hyperthyroid patients yielded almost no iodine in the supernatant organic solvent, but gave a protein precipitate which contained an excessive amount of protein-bound iodine.

Progress in this field is largely conditioned by the development of new techniques for studying dissolved colloids. It has been possible in a preliminary way (72) to fractionate plasma into characteristic protein frac-

tions and to compare the relative amounts of iodine which are contained in these fractions. The great bulk of the circulating iodine appears in the crude albumin fraction, namely, that portion of plasma protein which just escapes precipitation at half-saturation with ammonium sulfate. The fibrinogen and gamma-globulin fractions contain relatively little, and even the high albumin fractions are low in iodine.<sup>2</sup> On further purification, the maximum concentration appears in the alpha-beta globulin fractions. There is one slight complication in this scheme, namely, the appearance of a sharp maximum in the alpha-beta globulin fraction rather close to the lower albumin fraction. This sharp peak actually represents the highest concentration of iodine with respect to plasma protein. In terms of the total iodine found, however, it represents a relatively small amount of the total circulating material. In cruder fractions of horse serum the same general trend was found; and the result has since been confirmed (430) on more pure fractions furnished by Oncley (345) and Strong (483) working in Cohn's laboratory. Furthermore, in the ultracentrifuge the plasma iodine becomes more concentrated in the albumin layer (394).

From these results one must conclude that the circulating "hormonal" iodine is bound as a corporate part of plasma protein. For routine studies of serum "hormonal" iodine the total protein-precipitable "P" iodine is recommended by Salter (411). General methods may be used to separate this fraction, including heat-coagulation (417), precipitation with acetone (420,487), and precipitation with alkalinized zinc sulfate (288). The older methods required at least 10 ml. of serum, but the newer procedure (426) can be performed satisfactorily with only 1 ml.

There is still a controversy as to the presence of organically bound iodine in the red cells. Some workers (411,384) doubt its existence in normal erythrocytes, while others (305) have reported values of 6.4  $\mu\text{g.}\%$  of the plasma and 6.3 for the erythrocytes. As pointed out by Salter (415), for the present it is safer to consider only the plasma or serum protein-precipitable iodine in estimating the circulating "hormonal" iodine. Until this confusing point is permanently cleared up, it will be desirable to avoid the use of whole blood in iodine analysis. Furthermore, varying hematocrit readings in pathological conditions serve only to confuse the issue further by adulterating the plasma with red cells in varying degree. As far as serum and plasma are concerned, however, there is no appreciable difference; because (as already pointed out) the iodine bound in fibrinogen is only an extremely small percentage of the total protein-bound iodine.

With this background in mind one can proceed to set arbitrary normal values for man and other animals. The actual range of normal values accepted in any laboratory depends upon one's clinical concept of euthyroidism, and is a matter of personal impression. Also, minor variations in

the technique employed influence the values adopted. It should be noted also that the range for many laboratory animals is lower than for man. In the author's experience both in Boston, and in New Haven, an arbitrary normal range of some 4.0 to 8.0  $\mu\text{g. \%}$  suffices for diagnostic interpretation (414). Young people at puberty, when the basal metabolic rate may rise 25%, tend to have somewhat higher values, but rarely over 8.0  $\mu\text{g.}$  per 100 cc. of serum or plasma. A score of blood samples collected by mail from other sections of the country e.g., North Carolina, New York City, Philadelphia and San Francisco have fallen within approximately the same range (426), but these have no statistical validity. In another series in New Haven (398) there seemed to be an occasional value as low as 3.0 and an occasional value as high as 9.0. Obviously, one is dealing with the tapering extremes of population in such instances and the arbitrary limits of normality will vary from one investigator to another. The general mean of around 5  $\mu\text{g. \%}$  for normal adults, however, seems to be rather consistently encountered.

Other animal species are now being studied, with newer methods which require as little as 1 cc. of serum. Not enough data are available, however, to set these levels clearly. In the monkey (*Macacus rhesus*) the value seems close to that of man. In the horse and dog, it is distinctly lower than man, perhaps by 40%. In the dog, rat, mouse and domestic fowl values for "hormonal" iodine of 3 to 4  $\mu\text{g. \%}$  are usual (493). Indeed the apparently normal dog's value may fall as low as 2 (361).

In pathological human patients, however, quite striking deviations from the normal range occur. In long-standing myxedema, the value for "hormonal" iodine approaches nil as shown in Fig. 11. On the contrary, in pronounced hyperthyroidism, such as in early Graves' disease, values of 12  $\mu\text{g. \%}$  or higher are not unusual. In the so-called "thyroid storm" even higher values, 15 to 20  $\mu\text{g. \%}$ , are met. The term "thyroid storm" implies that the individual is carrying a rather high concentration of serum "hormonal" iodine and has finally become decompensated under this strain. Not all individuals decompensate at the same level of thyroid hormone concentration. Therefore, the plasma iodine concentration cannot be used to measure storm directly. Values above 20 in thyroid storm, particularly in women, are not unusual; the author has seen a value as high as 26  $\mu\text{g. \%}$  in a male case of thyroid storm with recovery. Much the same general range of "hormonal" protein-precipitable iodine has been reported by several investigators (32,394,304,276). A different technique involving organic solvents (e.g., acetone) has been employed by some investigators in such a manner as to split the "P" iodine. For example, Swenson and Curtis (487) obtained an average value in central Ohio for normal bloods of 0.88  $\mu\text{g. \%}$  after "acetone fractionation" as compared with 4.2 for the

value following "acetone precipitation." In appraising values obtained with the use of organic solvents, therefore, due attention must be paid to the analytical procedure employed and the presence or absence of red blood cells.

In simple goiter, such as is found in the adolescent girl without severe iodine lack, the values tend to be normal or on the low side (396).

Correlations between serum iodine and basal metabolic rate in several types of thyroid status show significant regression between these two variables in the hyperthyroid group ( $P < 0.01$ , 76 cases) (532). No significant regression was found in the hypothyroid group ( $P > 0.20$ , 18 cases). In the normal group (110 cases with total iodine determination and 59 cases with

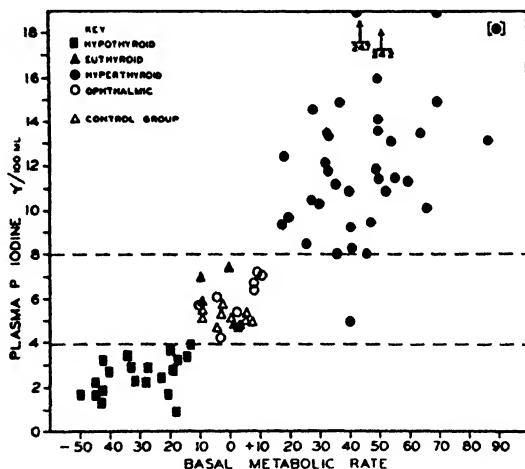


FIG. 11.—The serum "hormonal" iodine is a good index of the organism's supply of thyroid hormone. (From Salter and Bassett, *Trans. Assoc. Am. Phys.* **66**, 83, 1941.)

protein-bound iodine determination) there was a significant regression, but if the smaller sub-group of 59 cases with protein-bound iodine only are considered, the regression was not significant ( $P > 0.20$ ). An over-all analysis of regression in the three groups considered together showed that, although there was an over-all regression which could occur by chance less than one time in a hundred, there was significant variation between the slopes of the three graphs.

The serum precipitable iodine in nine cretin children was mostly below  $2.0 \mu\text{g. \%}$ , whereas only one of 72 euthyroid controlled children fell significantly below  $4.0$  (286). This one value of  $2.4 \mu\text{g. \%}$  was found in a cachetic child with anorexia nervosa, and rose to  $4.2$  when proper nutrition was established. In the cretin children the values increased rapidly when thyroid medication was administered. Blood taken from the umbilical cord of

two new-born infants showed a protein-bound iodine value of 7.3 and 10.0  $\mu\text{g. \%}$  respectively.

Very soon after thyroxine is injected into the normal individual intravenously, it will have become incorporated into the plasma protein. Thereafter the plasma cannot be distinguished from plasma which has become hyperthyroid naturally. The actual transfer of this hormone from the plasma into the tissue fluids, e.g., lymph, is a matter which has only recently been studied (426).

For some time it was supposed that the gland projected its hormone into the blood stream in the form of thyroglobulin. Attempts have been made both in the thyroid lymph (198) and in the blood of the thyroid veins to demonstrate thyroglobulin. In practically all instances, however, it has been impossible to show that thyroglobulin was present. For example, Lerman (255) studied human cases just after operation. He used a micro-precipitation technique involving immune serum. He was able to demonstrate thyroglobulin in the blood of the thyroid veins and also in the peripheral blood only after a rather drastic surgical procedure and then for only a short time. Carlson's experiments on the thyroid lymph (59) indicated the same result. In short, even as it leaves the thyroid gland, the circulating hormone apparently has lost its characteristic colloidal properties and is no longer recognizable as thyroglobulin. The split fragments formed from the storage depots of "colloid" may be similar to the polypeptide prepared (186) by the digestion of thyroglobulin with proteolytic enzymes. In any case, the split product is incorporated within an hour or two into the circulating plasma protein.

The tissues of normal animals contain a small amount of organically bound iodine, e.g. about 5  $\mu\text{g.}$  per 100 g. of muscle (304,306). When animals are deprived of their thyroid glands, a rapid increase in iodine excretion occurs, chiefly in the urine. Eventually, as the animal sinks deeper into hypothyroidism and myxedema, the iodine concentration in the muscles falls nearly to 1  $\mu\text{g. \%}$ . This low concentration of iodine may be mainly inorganic, but technical difficulties have not allowed this question to be settled as yet. On the contrary, in animals made hyperthyroid through the injection of thyroxine, the muscles are found to contain two or more times the normal concentration of iodine; and this iodine appears to be organically bound.

## VI. EUTHYROIDISM AND DYSTHYROIDISM

The estimation of thyroid function has been greatly clouded by the almost universal confounding of simple hyperthyroidism with the complicated syndrome of Graves' disease. For many years it was difficult to corre-

late metabolic findings with gross clinical phenomena. Consequently, cases of exophthalmic goiter which were called very "toxic" turned out to have surprising normal iodine metabolism. It is now recognized, however, that Graves' disease includes certain symptoms which are not specifically caused by hyperactivity of the thyroid.

In thinking of thyroid function it is desirable to view the situation from the standpoint of the peripheral tissue cells. If these cells enjoy a state of well-being with respect to thyroid supply, the organism may be considered "euthyroid." This state may be maintained, after the thyroid gland has been removed, through judicious medication supplied by a skilled physician.

Clinical data has shown conclusively that measurements of the organically bound iodine of the plasma afford rather accurate quantitative evidence of thyroid function. At least this appears to be true in man. For example, in a series of 70 cases ranging from severe myxedema to "thyroid storm," the coefficient of correlation between the basal metabolic rate (BMR) and the plasma "hormonal" iodine was  $0.82 \pm 0.05$  (417). These cases were selected because the bedside diagnosis and the BMR were compatible. In only one case was the iodine determination incompatible with the diagnosis previously established. Simultaneously, 29 other cases were accumulated in which the BMR did not confirm a well-considered clinical diagnosis. In this group the plasma iodine checked the physicians in all but one case. The data are summarized in Table VI.

A further source of confusion is the crudity of the determination of BMR. When large groups of normal individuals are studied, certain of them will be found with rates regularly well below -15; indeed occasionally as low as -25 or -30. Their circulating "hormonal" iodine is normal. In other words, their metabolism is normal but sub-standard. Possibly the usual statistical formulae for BMR based on surface area are inadequate; or perhaps the tissues of these individuals are accustomed to a somewhat lower than average resting caloric consumption. In these cases it appears logical to consider these persons as "euthyroid" because they are healthy.

The most frequent error encountered is the falsely high metabolic rate. This may be the result of nervousness on the part of the untrained patient or the result of cardiac decompensation or of fever. A recent meal will also contribute to the confusion.

The catalytic effect of iodide upon the rate of reduction of ceric ions to cerous ions (433) forms the basis of present micromethods (70), and has been employed by various workers (426,387,297). Care must be taken to exclude samples from subjects receiving exogenous iodine (309,429).

Human spinal fluid, which is normally nearly free of protein, is usually nearly free of iodine. Moreover, this small concentration of iodine frequently

approaches 0.5  $\mu\text{g}$ . % or less and probably is almost altogether inorganic. If the individual under study has been treated with high doses of iodide, presently the spinal canal will become suffused with iodide, although the concentration in the spinal fluid is ordinarily below that of the plasma (148). What significance this finding has in regard to the "blood-brain

TABLE VI  
SIGNIFICANCE OF SERUM "HORMONAL" IODINE IN EVALUATING NET  
THYROID FUNCTION\*

Clinical diagnosis	No. of cases	Iodine level	
		Compatible	Incompatible
<i>Compatible B.M.R.</i>			
Hypothyroid.....	20	20	
Hyperthyroid.....	38	37	1
Ophthalmic.....	8	8	
Euthyroid.....	5	5	
Control .....	10	10	
Total .....	81	80	1
<i>Incompatible B.M.R.</i>			
Hypothyroid.....	0	0	
Hyperthyroid.....	1	1	
Ophthalmic:			
Low BMR .....	5	4	1
High BMR.....	2	2	
Euthyroid:			
Low BMR.....	9	9	
High BMR.....	12	12	
Total.....	29	28	1
Grand total.....	110	108	2

\* From Salter and Bassett, *Trans. Assoc. Am. Physicians* 56, 82 (1941).

barrier" is unknown. The absence of protein-bound iodine in the spinal fluid is additional evidence in favor of the hypothesis (already discussed) that the hormone is distributed throughout the body bound to protein.

The concentration of iodine in milk seems to depend largely upon the concentration of iodide in the plasma. Early in the puerperium (301) the colostrum yields only 2 to 5  $\mu\text{g}$ . of iodine per twenty-four hours, at a concentration from 24 to 5  $\mu\text{g}$ . %. After lactation has been established, the concentration may amount to 8 to 45  $\mu\text{g}$ . % (117) and 20 to 47  $\mu\text{g}$ . of iodine

may be secreted within twenty-four hours. In a lactating woman the total monthly excretion of iodine apparently ranges from 2.5 to 3.5 mg. (510) when no particular effort is made to force iodine intake. When the casein and lactalbumin of the milk are coagulated, most of the iodine is precipitated with the protein coagulum, probably by simple adsorption (128). Robertson (404) concludes that normally there is a little thyroid hormone in milk, but not enough for normal maintenance. When the mother is made hyperthyroid, however, the nursling may also become thyrotoxic. In lactating cattle (132,211,468) the iodine in the milk varies with the ingested iodine, being increased by pasturage at the seacoast (437), and still more by deliberately reinforcing the fodder with iodine (438,176,386,240). This iodide is preponderantly found in the skimmed milk fraction, rather than in the butterfat (307). There is no evidence that the nursing infant receives any thyroid hormone by way of the milk so produced. Furthermore, no thyroxine-like fraction can be isolated from milk after hydrolysis. Under abnormal circumstances, when iodide in doses over a gram is forced upon the lactating mother, the concentrations of iodide in the milk may reach several milligrams per cent. The possibility that such concentrations might produce iodism in the infant is a cogent one for dairymen who feed iodide or thyroxine-like material. In terms of total metabolism, the cost of the increased milk production is high. Therefore, the economic soundness of this procedure awaits further study. Studies by Turner and his associates (510) indicate that the iodine concentration in the milk of cattle may reach values as high as 200  $\mu\text{g.}$  % when the fodder is reinforced with iodine-containing material. The normal iodine content of the milk is low, and it can be increased easily by feeding inorganic iodine or by the use of thyroprotein (506).

In regard to other body fluids the following trend seems to be maintained: the body fluid contains the same concentration of iodide as exists in the plasma, and in addition, a protein-bound moiety in proportion to the amount of protein which the fluid contains. This protein-bound moiety will be somewhat richer in iodine than the crude whole serum protein. Many exceptions, however, will be found, especially when the ingestion of iodine is being varied. For example, the bile tends to contain rather high concentrations of iodide which it abstracts from the portal circulation. In dogs (441) values ranging from 16 to 115  $\mu\text{g.}$  % have been reported. Indeed, after the administration of thyroxine intravenously, the bile may even contain active thyroxine-like material (542). Similarly, the thoracic duct lymph, which is particularly high in protein after a meal, may also contain rather large concentrations of iodide derived directly from the chyme. After the ingestion of iodine-containing fodder, rabbit's bile may reach 55  $\mu\text{g.}$  % of iodine within three hours. In the saliva the concentration

depends largely upon recent ingestion. In the fasting individual it may be less than one  $\mu\text{g. \%}$ , whereas after the therapeutic administration of iodide, concentrations as high as 362  $\mu\text{g. \%}$  (446) may appear within a few minutes. The iodide concentration appears to be much the same as that of the serum (55).

## VII. The Effects of Thyroid Hormone

As yet there is no clear picture of the ultimate function of the thyroid hormone. Perhaps it is part of a super-enzyme system in tissues which controls the organization and activity of lesser enzyme systems. Certainly it can alter the apparent concentrations of oxidative (71) and of hydrolytic enzymes in cells (436). Possibly it serves as an intermediary between the organizers of Spemann (477) and the humbler catalysts. Is thyroxine the essential prosthetic group of such a specialized catalytic mechanism? If so, with what acceptor and co-enzyme is this "thyro-enzyme" associated? Such questions can not be answered at present. We know the thyroid hormone's function only by the manifold distortions of metabolism and body structure which characteristically accompany thyroid deficiency or excess of thyroid.

### A. THE RESULTS OF HYPOTHYROIDISM

The functions of the thyroid hormone may be classified conveniently under two main headings: the maturity function and the "spendthrift" function. The first of these is best seen when too little hormone is present, as in clinical myxedema and cretinism; the second in cases of over-function of the gland, as in toxic nodular goiter.

The classical experiments of Gudernatsch (162) demonstrated that baby tadpoles could be changed prematurely into baby frogs not much larger than flies, by adding an excess of thyroid hormone to the liquid medium in which the tadpoles lived. At the same time he showed that an excess of pituitary hormone would produce giant tadpoles, which, however, remained infantile. In the clinic one encounters all stages of retardation in development both mental and physical (including sexual), depending upon the degree of thyroid lack. In complete lack of thyroid beginning in fetal life, the result is the characteristic picture of cretinism.

Experiments by Barrnett (27) on cretinoid rats born of mothers treated with thiouracil have shown that the hypothyroid fetus suffers *in utero* from the following:

1. Myelination is delayed and is diminished in degree.
2. The appearance of classical tracts of fibers, both in the higher brain and in the spinal cord, is delayed.

3. The maturation of nerve cells throughout the central nervous system is delayed and impaired.

When the thyroid has failed entirely in adult life, the subject sinks gradually into myxedema. In the early stages, however, the classical picture is not evident because the individual at first suffers simply from partial hypothyroidism. It is very essential, therefore, to distinguish between the term *hypothyroidism* and *myxedema*. Hypothyroidism is a functional state. Therefore, in general, one should qualify it with the adjectives "mild" or "severe". It does not imply a visible morphological change, but can be described only in quantitative terms, namely, by the measurement of basal caloric consumption or of the circulating "hormonal" iodine. When hypothyroidism has been in existence for three months to a year or more, the skilled clinician will suspect the presence of beginning myxedema with its classical structural changes.

The use of basal metabolism as a gauge of thyroid function has led to the wide-spread misconception that its maturing and calorigenic actions are inseparable. Of special interest in this respect are the observations of Dodds and his associates (97), who administered dinitro-o-cresol in human myxedema. With this drug they were able to raise the basal metabolic rate from approximately *minus* 18 to approximately *plus* 73 in 5 days. Despite this large increase in energy turnover, however, the patient remained obviously myxedematous. This patient subsequently was maintained at +20 to +30 on 150 mg. of the drug daily without losing his myxedematous appearance. He responded promptly to thyroid, however, in characteristic fashion. Therefore the calorigenic and maturity effects of thyroid hormone are both secondary to some common result of thyroid action, but are independent of each other.

The histological picture of the thyroid in myxedema is extremely constant and characteristic. The parenchyma disappears and its site is invaded by phagocytes which remove the dying cells and the colloid from the follicles. There is an increased infiltration with lymphocytes and ultimately the whole region is replaced by scar tissue. During the course of the fibrosis, however, which presumably may last several years, a biopsy may disclose islands of apparently actively functioning follicular tissue even though the general disease is well advanced. The other endocrine glands show no very constant change.

A special type of myxedema, however, is seen in cases of Simmonds' disease. These cases are suffering from primary pituitary destruction or atrophy and the myxedema is secondary ("Simmonds' disease masquerading as myxedema" (315)). In these instances marked atrophy of the pituitary occurs characteristically. In some cases the pituitary is cystic; in others it has been replaced almost completely by scar tissue (127). Although

in animals the degree of adrenal hypertrophy is parallel to the thyroid's activity (206,268), clinical autopsies of Simmonds' cachexia do not bear out this finding with any constancy. Furthermore, the thyroid and gonads and the pancreatic islets show no characteristic findings.

The skin in classical myxedema has been studied carefully by Reuter (387). The significant changes include a hyperkeratosis of the epidermis; degenerative changes in the epidermal cells; pronounced edema of the corium; with separated elastic fibers and mucinous material in considerable quantities. The muscles of the heart and the general skeletal muscles are characteristically pale and edematous. The fibers are separated by edema fluid and the striations are poorly marked. In the gross, this often gives a false impression of hypertrophy. The myocardium is frequently very soft and pliable; there may be superimposed degeneration of blood vessels; and arteriosclerosis is not uncommon. This is true also for the brain, and may involve the cerebellum. Other parenchymatous organs show no characteristic changes, although there may be some tendency towards increased fat. The skeleton tends to be more heavily calcified than normally, as one might expect from the low calcium turnover.

#### B. HYPERTHYROIDISM AND REPLACEMENT THERAPY

The response of the myxedema patient to thyroid therapy gives the best picture available of the natural function of the thyroid hormone. The thyroid secretion influences cells in every part of the body. Of greatest interest historically is the effect upon total metabolism. Ordinarily this is measured at basal levels, but actually the BMR gives only a partial picture, because the thyroid hormone stimulates not merely the idling or resting metabolism but also increases the amount of spontaneous activity. Means (317) has described three metabolic levels of living for man, i.e. (a) the normal level at 0 basal metabolic rate; (b) the pituitaryless level at about minus 25; and (c) the thyroidless level at about minus 40. Actually, the discrepancy is greater than this because of the effect on total metabolism which is not susceptible of easy measurement. When the thyroid is removed totally from an adult, the basal metabolism declines in accordance with a semi-logarithmic curve (318), i.e., the stored hormone in body tissues is used up in accordance with an inverse compound interest law; day after day the same percentage of what is left is destroyed.

This point was first elucidated by Magnus-Levy (48) in the last century. After total thyroidectomy for as long as two months it may not be apparent, even to a good clinician, that the patient is hypothyroid, and it may be over a year before one can make the diagnosis of myxedema without hesitation. Conversely, in the treatment of myxedema one may relieve the hypothyroidism rather rapidly; but one cannot produce a morphological meta-

morphosis in a short time; in treating a myxedema patient, one literally starts with one creature and ends with another. It is possible to elevate the metabolism faster than the tissues can change, with the result that one may produce functional decompensation, as the metabolic rate overshoots its normal range. Cf. Fig. 12.

In myxedema the endogenous protein metabolism, as all other metabolism, is reduced. This is true if the usual mixed diet is not interrupted. In fasting rats, however, H. D. Hoberman has found with stable isotopic nitrogen that the basal nitrogen turn-over is characteristically retarded in

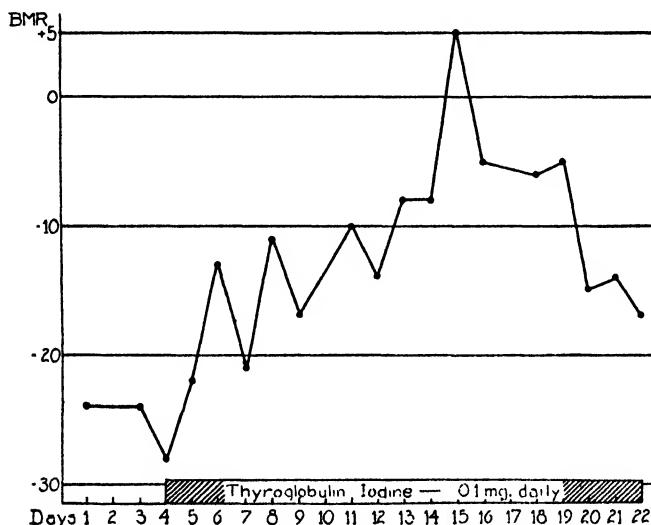


FIG. 12.—Response of human myxedema (surgical) to human thyroglobulin, illustrating the overshoot of metabolic rate during the relief of myxedema. (From Eppinger and Salter, *Am. J. Med. Sci.* 190, 653, 1935.)

marked hypothyroidism. In addition there is a retention of nitrogen which goes into the formation of the mucoprotein in the myxedematous fluid (49). When thyroid is given, this large amount of extra protein is changed into urea and excreted. Consequently, under therapy there occurs not only a water diuresis but also an increased excretion of urea, sodium chloride, etc. Some light on this phenomenon is thrown by the observation that thyroidectomy in animals led to a change in the secretion of the parotid gland (205). Ordinarily this secretion is serous in character, but as hypothyroidism develops, the secretion becomes mucous.

The diuresis of myxedema responding to therapy may also lead to an increased excretion of potassium and phosphate which are traditionally connected with the breakdown of protoplasm. Undoubtedly, as the myxedematous process is reversed, profound changes do occur within cells as

well as outside of them. The reduction of effective blood volume by 25% during marked hypothyroidism is only one indication of a significant alteration in the distribution of body water and its solutes. Obviously, one function of the thyroid hormone is a continuous control of fluid balance in the organism. The blood globulin is increased and accordingly the total blood protein is moderately elevated (98). The spinal fluid protein is likewise higher than normal (498). The total number of red cells is reduced characteristically, but the cells are often well filled with hemoglobin, and the color index is frequently normal, as in pernicious anemia (which may be suspected erroneously). Similarly the blood cholesterol is elevated to values between 300 and over 500 mg. per 100 ml. of blood (150,214,362), whereas 150 to 230 (360) are found in normal adults. The significance of this fact is not understood. Strangely enough, occasional cases of true myxedema turn up with a normal blood cholesterol. These may be associated with malnutrition or liver disease.

Two special types of metabolic balance studies have been made in myxedema. The one concerns creatine metabolism; the other the metabolism of iodine. The iodine excretion is rather low in the urine of myxedema patients, presumably due to a lowered food intake (74,443), and the excretion will rise markedly if extraneous sources of iodide be supplied. Immediately after thyroidectomy there is a large increase in iodide excretion, because the iodine concentration in the tissues at large is falling and there is no thyroid gland to salvage the split products of the tissue hormone. Of course, the plasma precipitable iodine falls markedly in myxedema.

The studies on creatine metabolism are of considerable interest, because they harmonize with similar studies in hyperthyroidism. No creatinuria occurs in myxedema patients, and the retention of creatine on feeding this substance is about normal or slightly higher than normal (460,502). When thyroid is given, however, as the metabolism rises there is a prompt appearance of creatinuria, reflecting a loss of creatine tolerance (389). As shown in Fig. 13, this phenomenon reaches a maximum within a week and then all but disappears, as the metabolism assumes a steady state at a higher level. In other words, creatinuria appears whenever the patient is elevated to a higher level of metabolism. Presumably this is connected with the tissue metamorphosis and related to the classical disorders of muscle metabolism which Askanazy (18) described. As might be predicted, iodide relieves the creatinuria of Graves' disease but not that induced by the feeding of thyroid substance. In the ordinary tolerance test about 2.6 g. of creatine is administered, an amount which implies the subsequent excretion of 2 g. in the urine. For normal adults the anticipated excretions are over 80% for men and over 70% for women. In thyrotoxicosis the values tend to lie below 60%.

It was recognized in the last century that in myxedema a considerable

amount of salt solution accumulated in the organism. When the patient responded to thyroid, one of the classical effects noted in 1893 was a diuresis which freed him of the extra fluid together with the dissolved electrolytes (252). As the metabolic rate increases, in addition to this loss of fluid by way of the urine, another increase in fluid loss is now known to occur through the lungs (160). These water losses influence the volume and properties of the body fluids (93,496). In addition, in the absence of the thyroid, the central nervous system, the skeletal muscles and the blood show a defi-

**CREATINE TOLERANCE TESTS  
IN MYXEDEMA  
DURING THYROID MEDICATION**

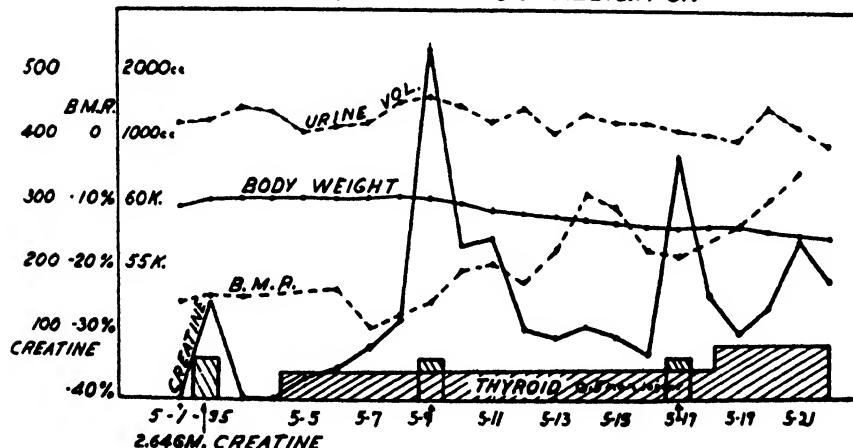


FIG. 13.—Case E. W., demonstrating the effect of thyroid administration upon creatine excretion, basal metabolic rate, alteration in weight and urinary output. Creatine tolerance tests revealed a marked decrease in creatine retention after thyroid therapy. A sudden increase in thyroid dosage resulted in an increased creatinuria. (From Thorn, *Endocrinology* 20, 633, 1936.)

nite anhydremia (174). Similarly, the administration of thyroxine causes water and NaCl to pass from the tissues to the blood, thus increasing the total blood volume over 30% (142). The increased serum viscosity in hypothyroidism probably is related to a higher globulin:albumin ratio than normal. Thus in hypothyroidism there is a general tendency toward plasma dehydration, while in hyperthyroidism the reverse is true. It is clear that the thyroid hormone exerts a marked influence upon the distribution of electrolytes and liquid among the various body tissues and the blood.

The total magnesium content of the serum is essentially normal (517) (34) in thyroid disturbance, averaging 2.5  $\mu\text{g. %}$  (472). When the serum is subjected to ultrafiltration, however, an interesting phenomenon is dis-

closed (471). In myxedematous patients and in totally thyroidectomized dogs, nearly 100% of the serum magnesium is ultrafilterable. In normal individuals, the value lies between 78 and 97% and in hyperthyroidism between 38 and 79%. These results suggest that in thyrotoxicosis the patient's body fluids are deficient in magnesium ions. Possibly this feature contributes to the hyperirritability of the nervous system in Graves' disease.

A low thyroid activity also favors the storage of calcium in human bones, whereas a high thyroid activity favors its removal (23). Moreover, (8), in Graves' disease the high calcium excretion can be reduced to normal when treatment with iodide has produced a remission of the hyperthyroidism. In long-standing severe cases of Graves' disease osteoporosis occurs because of depletion of the skeletal lime salts (210). The increased calcium excretion occurs despite a normal level of calcium in the blood. In hypothyroidism, urinary calcium is greatly diminished, and approximates the fecal level. The total amount excreted by an adult may be as little as 40 mg. of calcium per day. Severe hyperthyroidism does not produce osteoporosis in rats, provided that the diet is adequate in calcium (467). Drill (104) concluded that the loss in calcium from bones, e.g., 2.7% from the femur, merely reflected the general loss of body weight, e.g., 23% in his severely hyperthyroid rats. It is still a moot question whether the mechanism of mobilization involves an associated hyperparathyroidism, as favored by Hansman and Wilson (177). When both parathyroid and thyroid are lacking in man, both hormones may be needed to restore the very low serum calcium to a normal concentration (22). In infancy hypothyroidism leads to a dense calcification of the epiphyseal plate in a long bone, e.g., the radius (537); and this "line" is dissipated by administering thyroid. In early childhood, ossification is delayed and finally appears in multiple, irregular foci. The effect of thyroid therapy on bone growth is reflected in the serum phosphatase activity. The value is low in cretins, but increases to normal when adequate thyroid is given (491). Not only the long bones, but even the teeth are affected. Also in newborn rats the time of eruption of the incisor teeth is shortened by the administration of thyroxine. Probably these effects are comparable to the general influence of thyroxine on all growing tissue,—including mouse fibroblasts in tissue culture.

It is quite clear that the concentrations of serum cholesterol, phosphatides and fatty acids are influenced significantly by thyroid hormone. In 29 patients before, during, and after treatment with thyroid, the titrated fatty acids and the cholesterol in the serum were closely correlated (149), as were also the lipoid phosphorus and the cholesterol. In the hypothyroid range, the lipoid phosphorus ranged from about 6 to 24 mg. %, and the titrated fatty acids from 5 to 33 milliequivalents (149). In the hyperthyroid

range similar correlations were found. The level of serum lipoids, however, was not obviously related to the BMR, and was not of great value in predicting the degree of improvement after thyroidectomy. Changes in the cholesterol were accompanied by proportionate changes in phospholipids, and to a lesser extent in fatty acids. In patients not suffering from hyperthyroidism, the addition of iodide causes little change in blood lipoids (509) whereas in Graves' disease a rise in serum cholesterol may result from therapy with iodide.

Because it is well demonstrated (37, 92) that there is a roughly inverse trend between thyroid activity and the concentration of serum cholesterol, this determination has been used extensively as a confirmatory diagnostic test in the clinic. In myxedema it is usual to find values above 300 mg. % and in hyperthyroidism the value for cholesterol tends to be reduced, although this reduction is less spectacular. Nevertheless low values have been encountered consistently both in thyrotoxic patients (217) and in animals rendered hyperthyroid experimentally (444). If one knows the normal value for a given patient, the alterations in cholesterol with therapy afford a rather constant and useful criterion, but the normal values for random patients vary rather widely. Furthermore, the methods used clinically are somewhat crude because they measure other substances besides cholesterol. For that reason each laboratory must state its own normal range (212,213,214,216,149,285).

During experimental hyperthyroidism the requirements for vitamins A, C, and part of the B complex are increased, and part of the effects of thyroid feeding previously ascribed to thyroid *per se* are due to a relative deficiency of one or more of these vitamins (105). In patients with myxedema blood levels of carotene are higher, and the vitamin A levels lower than normal (105). Recently thyroidectomized rats have been shown to metabolize carotene to vitamin A very poorly, and if carotene is the only source of vitamin A in the diet, signs of vitamin A deficiency will appear (106). More precise data concerning vitamin metabolism in hyperthyroidism will be discussed presently.

Certain biophysical measurements have been made which are of interest although still in a premature state. The electroencephalogram in myxedema, for example, shows a characteristic slowing of the alpha-wave rhythm. In normal people (406), the alpha-waves recur at a rate of approximately 8 to 11 per second. In frank myxedema the rate tends to be below 7, and in hyperthyroidism as high as 14. Furthermore, the reaction time of the myxedematous patient is prolonged. Whereas the normal individual reacts to a standard stimulation in approximately 0.23 of a second, the profoundly hypothyroid individual may take 0.5 of a second longer to respond (147). In hyperthyroidism the corresponding value is slightly lower than normal, i.e., about 0.2 of a second.

Another interesting measurement is the so-called impedance angle (53a, 53b). When alternating current is passed through the skin of a myxedematous patient the impedance is increased, presumably due to poor circulation through the integument. After thyroid therapy the phase angle diminishes, and in hyperthyroidism is less than normal. These are but examples of a number of biophysical phenomena which have been studied in hypothyroid patients.

### C. THE RESULTS OF HYPERTHYROIDISM

The pathology of the hyperthyroid gland has occasioned a great deal of discussion chiefly because the element of time and the chronicity of the picture often have not been evaluated adequately. These features have already been considered elsewhere. Many of the characteristic changes may be complicated by a scarcity of iodine, or by variations in the natural supply of ingested iodine.

A persistent thymus occurs in about half of the cases of Graves' disease. Thus far there is apparently no established abnormality of the parathyroid or pituitary glands. Extensive discussion has occurred with regard to the suprarenal gland in Graves' disease. Indeed Crile (85) recommended its removal (unilaterally) in hyperthyroidism. On the other hand Marine (291) and Holst (204)—the latter on the basis of autopsy studies—described a hyperplasia of the adrenals, which suggested that these glands were under constant strain in the disease. The other organs show no regular abnormality. The heart shows no obvious gross change, despite the great emphasis in the clinic upon the extra load which a hyperactive thyroid may throw upon an already damaged heart.

White (526) has presented four theories of heart disease. First, the general increase in body metabolism places an extra load upon the heart and may produce a work hypertrophy, followed by a dilatation due to cardiac decompensation, and ultimately to various arrhythmias accompanying myocardial failure. A second theory is that the heart itself is a seat of specific thyroid stimulation, (*cf.* 215): so that a characteristic "toxic" effect is produced upon the myocardium. In addition, Boas (43) has suggested that the tremendous engorgement of the thyroid acts as a sort of arteriovenous shunt which increases the burden of the heart and forces enlargement. The last, is that a natural thyrotoxic degenerative change occurs in thyrotoxicosis and *per se* leads to cardiac dilatation and failure. In general, however, cardiac enlargement does not occur in thyrotoxicosis without complicating factors, like long-continued auricular fibrillation or hypertension. In an extensive study of the pathology of hyperthyroidism, Lewis (262) concluded that there was no evidence of a characteristic primary myocardial lesion; but that the lesions found associated with thyrotoxicosis were due to rheumatic fever, arteriosclerosis or coronary sclerosis. Acute heart failure has

been produced in animals by the administration either of thyroxine or of thyrotropic hormone. The latter instance is the more interesting, because it represents heart failure produced by hyperactivity of the animal's own gland. Thus Loeser (270) and Elmer (118) by giving large doses of thyrotropic hormone to guinea pigs were able to produce first tachycardia, then fibrillation and finally heart block with heart failure. This decompensation was characterized by low electrocardiographic potentials and massive edema of the animals. A final dilatation of the left heart led to death. This demonstration that an animal can be killed in a few weeks by the administration of thyrotropic hormone is one of the most lucid pictures of the pure disease that we have. Furthermore, when rats are made thyrotoxic by the injection of thyroxine, a peculiar block of the vagal (i.e., muscarinic) effect on the heart occurs (203). As a result, vagal stimulation causes the heart to accelerate. This very interesting paradox suggests that large doses of thyroxine may have an action related to that of ephedrine in connection with sympathetic stimulation. These observations might offer a clew to the mechanism by which the heart is constantly accelerated in thyrotoxicosis, and thus overtaxed.

Two tissues seem to merit special interest in hyperthyroidism, namely, the liver and the skeletal muscles. In marked hyperthyroidism, there is a long tradition that liver damage may ensue. This applies both to Graves' disease (519) and to toxic nodular goiter (33).

It may, however, be doubted whether there is anything specific about the liver damage which occurs in Graves' disease. Rather, it may be simply the result of an overstrained metabolism with particular emphasis upon lack of adequate nutrition. Many clinicians who treat hyperthyroid patients are not greatly impressed with the importance of liver changes in a routine thyroid clinic. Furthermore, patients dying incidentally in the course of hyperthyroidism do not show characteristic liver lesions consistently (262,313). Increased bilirubin (538) and impaired liver function (353) have been reported. Various sugar tolerance tests have been used to indicate liver abnormalities. For example, the curve of galactose in the blood after oral administration was found rather high in patients with hyperthyroidism (12).

In 16 of 20 cases there was moderate impairment of liver function as indicated by the oxidation of cinchophen, but not severe disability (263). Likewise, of 148 hyperthyroid patients, the hippuric acid test gave normal values in only 18 (28). Lord and Andrus (275) after injecting 2-methyl-1,4-naphthoquinone intravenously found that plasma prothrombin did not rise significantly in patients with intrahepatic jaundice; whereas it did rise in cases of extra-hepatic jaundice. Accordingly, these authors studied 36 cases of hyperthyroidism and discovered a significant fall of prothrombin

after operation in 29 of the 36 patients with toxic goiter. In animals various studies have been made of the increase in hepatic size and changes in liver glycogen following the administration of thyroxine. Daily subcutaneous injections of 4 mg. of thyroxine to rabbits until death at the end of five days, caused extensive degeneration and necrosis (145). Prolonged feeding of thyroid extract to dogs caused a marked increase in bromosulfalein retention, especially if the amounts of yeast in the diet were reduced (107). Many other studies of the liver under laboratory conditions have been made, including increased susceptibility to chloroform and carbon tetrachloride (311), to intercurrent infections and to the deleterious effects of anoxia (311).

The skeletal muscle is an undoubted site of disturbance which is nearly specific in character (18). Patients with severe hyperthyroidism complain of mild muscular weakness or muscular atrophy. The more severe manifestations of this disturbance even suggest a muscular dystrophy (25). The muscular weakness may be so severe that the patient cannot be relied upon to stand unsupported. While most normal people can extend a leg horizontally for over a minute, the thyrotoxic patient frequently cannot endure half that time (245). The myasthenia may be so marked as to mimic myasthenia gravis or even acute bulbar palsy. When protracted, occasional cases show so much muscular wasting as to simulate progressive muscular atrophy.

The high caloric consumption in hyperthyroidism is classical and was well known in the last century. It is less well appreciated that the efficiency of muscular work is lowered in thyrotoxicosis, (54). In thyrotoxic patients, muscular activity entails an oxygen consumption of nearly 40% more than normal. Such patients are particularly sensitive to epinephrine and, when frightened, they become even more inefficient. The specific dynamic action of protein is superimposed upon this high caloric turnover, as shown by Aub and Means (24).

It has already been pointed out that the thyroid hormone has both a "metamorphosing" and a "spendthrift" effect. There are certain striking or paradoxical distinctions between these two effects. For example, in growing thyroidectomized rats, a moderately large dose of thyroxine may bring about the continued growth of the rat with the attendant storage of nitrogen, fat, and other constituents of bodily tissues, including the skeleton. On the contrary, the same dose in the thyroidectomized mature rat may lead to progressive weight loss until emaciation is produced. Similarly tadpoles show prompt metamorphosis when treated with either thyroxine or acetyl thyroxine (231,488). When these compounds were administered quantitatively by injection into the body cavities of giant tadpoles, those injected with thyroxine lived only a short time after metamorphosis,

whereas those injected with the acetyl derivative developed into miniature frogs. On the other hand, the acetyl derivative was found to have no influence upon the metabolism of the normal human being, whereas thyroxine, of course, produces typical hyperthyroid effects. Therefore, although the production of heat is markedly increased after the administration of thyroid hormone, (144) it seems reasonable to conclude that the increased metabolism, *per se*, is not the cause of metamorphosis. This conclusion is substantiated by the observation (97) that the basal metabolism of the myxedematous patient can be raised approximately to plus 73% without alteration in the appearance of the patient. Strictly speaking, one might take the viewpoint that the process of metamorphosis regularly continues, although in a minor degree, up into the hyperthyroid range; and accordingly the hyperthyroid patient would be expected to show small structural differences which are abnormal extrapolations of normal metamorphosis. For example, the amount of mucoprotein in the skin progressively decreases as the metabolism is raised above normal. From a practical standpoint, however, one might say that from -50 to -20% in adult human patients, the chief effect of the thyroid hormone is structural; whereas above minus 20 the chief effect of the thyroid hormone is catabolic. The latter effect is accompanied eventually by various structural changes. For example, there is a loss of flesh, of fat, of fluid, of salt, and even of skeletal components among other features of this catabolism. With respect to carbohydrate metabolism, there is a clinical tradition that increased thyroid activity produces diabetes mellitus (cf. 221). On the other hand, only 1.1% of patients with hyperthyroidism have true diabetes (527). Recently Houssay (207) has shown that permanent diabetes can be induced in experimental animals under laboratory conditions. This permanent diabetes, which Houssay terms "metathyroid" diabetes, is of considerable theoretical interest, in the light of control experiments with alloxan. It remains to be proved, however, that these excellent experiments bear directly upon the origin of clinical diabetes.

Toward the end of the last century, important observations were made on the nitrogen metabolism of patients with thyroid disease. Friedrich Mueller (330) and Magnus-Levy (282) were impressed with the difficulty of maintaining nitrogen equilibrium in their hyperthyroid patients. For example, Mueller showed that although the nitrogen intake was increased to 52.6 g. daily, the output at this time was 57.3 g. In short, the mere elevation of protein intake could not achieve nitrogen equilibrium, because the specific dynamic action of the protein diet overbalanced any possible benefit. Later, however, it was possible to achieve nitrogen equilibrium through the sparing of protein by large intakes of fat and carbohydrate, which were sufficient to cover the total caloric turnover. In 1893, also,

Mendel (323) administered an extract of thyroid to a case of myxedema and showed that the daily excretion of urea increased from 14 to 36 g. Even under such conditions, however, there is an element of anabolism present, for Janney and Henderson (220) demonstrated a retention of nitrogen in a child suffering from hypothyroidism, following the administration of small amounts of thyroid hormone. Consequently, the total balance depended upon the balance between these two contradictory effects of the hormone. Ultimately Steyrer (481) was able to achieve nitrogen equilibrium in hyperthyroidism by increasing the protein content of the diet slightly and, at the same time, giving sufficient calories to meet the increased caloric turnover. Presumably the glycogen of the liver serves this same function for a very short time. Furthermore, if exogenous fat as well as exogenous carbohydrate be supplied, body proteins may be spared. In fact, no one class of food stuff is spared by thyroid activity (48). If sufficient carbohydrate and fat be supplied, however, a protein intake of one or two g. per kg. of body weight suffices to maintain equilibrium. Boothby and his associates (112) (50) studied the so-called "deposit protein" which had been emphasized by Lusk (279). By administering thyroxine to a normal person they decided that at least 16% of the body weight could be accounted for by the nitrogen so lost, together with its combined fluid. In crude language, therefore, it could be assumed that the normal person wears under his skin a sort of blanket of mucoprotein in solution. In myxedema this blanket becomes greatly thickened. In hyperthyroidism, it is worn thin. Whenever there is a change of metabolic level due to alterations in thyroid activity, the disintegration of this blanket will produce an outpouring of nitrogen which in itself need not necessarily mean that vital protoplasm was destroyed (but cf. 213). It is clear, that part of the metabolic effects on nitrogen metabolism produced by the thyroid should be considered as structural changes connected with metamorphosis rather than due to the obligatory burning of protoplasm. In addition, large doses of iodides can cause a 27% increase in the daily nitrogen excretion of dogs (157). This increased excretion does not occur after thyroidectomy. This constitutes a further demonstration that the thyroid is concerned with the mobilization of nitrogen apart from its effect on the basal metabolic rate.

It has been demonstrated by observations of Shorr (460) and Thorn (502) on creatine tolerance that this function is related to various levels of thyroid activity in man. Indeed, the significance of creatine excretion was studied early in this century because it seemed to constitute a good index of the destruction of tissue, particularly muscle (239,350,219). In hypothyroidism creatine might not be excreted at all, or in only very small amounts. Because skeletal muscle contributes about one-half of the body weight, it is not surprising to find that when wasting of this is induced

by thyroid activity, there ensues an excessive excretion of creatine, one of its characteristic chemical constituents. The creatinuria found in thyrotoxicosis is of peculiar interest because it constitutes a metabolic derangement of extra-thyroidal tissues which is highly characteristic for this disease state. Since Shaffer's classical observations in 1908 various attempts have been made to elucidate the mechanism of the disturbance. It seems clearly related to phosphocreatine metabolism in skeletal muscle, but appears not to involve smooth muscle or myocardium. Thorn and Eder (503) have compared it with muscular dystrophy, as did Ayer, Means, and Lerman (25). Even attacks of periodic paralysis occur, but without a characteristic derangement of creatine metabolism. The creatinuria disappears under treatment with iodide or thiouracil, if the etiology is thyrotoxic (459,461). Occasionally the myasthenia of Graves' disease responds to neostigmine but not usually. Why the creatine-creatinine disturbance occurs, no one knows. The spontaneous creatinuria, together with the impaired tolerance and low creatine index, all suggest a fundamental derangement of enzymic processes within skeletal muscle. Methyltestosterone produces increased creatinuria, but this is accompanied by an excretion of glycocyamine, which does not occur in thyrotoxicosis. Normally about one-fifth of the store of muscle creatine is turned over daily and must be replaced. When this process is impaired, not enough phosphocreatine can be formed from the dephosphorylation of adenosine triphosphate to supply an adequate source of higher energy. Apparently in Graves' disease, not enough creatine is assimilated; and in addition possibly the restoration of the adenosine-triphosphate-phosphocreatine cycle is interfered with, specifically. Moreover, the disturbance is almost universally present in patients suffering from undoubtedly hyperthyroidism.

Elsewhere a large section of this article is devoted to the problem of iodine balance. Here it should be pointed out, however, that the total excretion of iodine does not reflect precisely the amount of iodine given as thyroxine in acute experiments. It is true, that within a week after the injection of a large dose of thyroxine about three-fourths of the iodine can be recovered from the excreta (442). The net balance of iodine, however, will be determined by at least two factors, (a) the efficiency with which the thyroid salvages the breakdown products of iodine metabolism, (b) the amount of iodine released by the destruction of tissue. For example, if in the course of hyperthyroidism a large amount of muscle is destroyed, the natural iodine content of this flesh will be released and much of it eliminated. A similar loss of protoplasmic iodine occurs when an animal is thyroidectomized, because the stores of thyroid hormone in the tissues at large are slowly being used up, and the iodide which is liberated by the disintegration of tissue hormone ("thyrenzyme") is no longer salvaged by the

thyroid gland (484). Consequently, a temporary negative balance of iodide occurs, which is corrected only when the tissue reservoirs of iodine had been depleted.

In tissues there are at least two types of iodine, tissue iodide which is in equilibrium with the circulating iodide of the body fluids (516,415) and organically fixed iodine. Not much is known about this latter. In hyperthyroidism it increases and in hypothyroidism it decreases. McClendon and his associates (306) have suggested also that it is higher in those animals which have a more rapid metabolism per kg. of body weight or of active protoplasm. In certain organs like the ovary and the pituitary, it was formerly believed that a marked concentration of the organically bound iodine occurs, and that in hypothyroidism this concentration tends to drop markedly; but probably the high results formerly reported were analytical artifacts. Nevertheless, there is organically bound iodine in normal endocrine glands, e.g., the ovary and pituitary; and after thyroidectomy this iodine fraction tends to disappear. Whether it represents an accumulation of thyroid hormone, as suggested by Courrier (80), is not known.

Hunter (210) has called attention to the so-called "osteoporosis" which occurs not infrequently in neglected cases of severe Graves' disease. The basis for this is the high excretion of calcium, particularly of fecal calcium. Even urinary calcium, however, was increased 231% when the basal metabolic rate was increased only 55% above the normal average (23). It does not matter, apparently, whether the increased thyroid hormone is exogenous, or whether it is derived from a primarily hyperplastic gland as in Graves' disease, or from a toxic nodular goiter. Moreover, in myxedema an unusual storage of calcium may occur in the skeleton (8). In Graves' disease, when iodide is administered, the excessive excretion of calcium may disappear. All investigators agree that no appreciable alteration in the concentration of serum calcium occurs, despite wide fluctuations in the metabolic rate or in the concentration of serum "hormonal" iodine. Whereas in hyperparathyroidism, the increased secretion of calcium is mainly urinary, in hyperthyroidism both the urine and the feces contribute to the increased excretion; although the feces to a lesser extent. This fact alone suggests that the increased excretion in Graves' disease is not due to an associated hyperactivity of the parathyroid.

It has long been recognized that one of the early symptoms of hyperthyroidism may be an increase in bowel activity. Contrariwise, certain cases of constipation have been relieved by the administration of thyroid. After the administration of thyroxine to the rat the intestinal absorption of galactose, glucose, xylose, starch, and oleic acid was increased, and after thyroidectomy the absorption of glucose was decreased (10, 11). When thyroxine or castor oil or cascara sagrada were administered to rats,

the fecal excretion was doubled. Conversely, the fecal calcium of hyperthyroid rats could be minimized by restricting the intake of food or by injecting morphine. In other words, thyroxine could influence the activity of the digestive tract by increasing the intestinal absorption of substances susceptible of phosphorylation and by increasing the gastrointestinal motility. The altered intestinal absorption of sugars and starch may account for the apparent reduction of the sugar tolerance in hyperthyroidism and the apparent increase of sugar tolerance in myxedema. For example, Althausen pointed out that in studies of 121 control subjects, compared with 130 hyperthyroid patients and 6 patients with myxedema, distinctive results comparable in diagnostic utility to those obtained with the BMR could be established (10,11). In rats in which the BMR was increased more than 50% by the administration of thyroxine, he found a marked acceleration of the absorption of sugars and starch in the digestive tract. In thyroidectomized rats, however, in which the BMR averaged minus 43% the absorption of glucose was reduced from 171 to 91 mg. The calcium exchange of the intestine also was studied. The basic diets used were nearly free of calcium and could be reinforced with calcium lactate at will. The results showed that the absorption of calcium was not diminished in hyperthyroid rats fed an abundance of calcium lactate. On a calcium-free diet, however, the fecal output of calcium in hyperthyroid rats was twice the normal. Nevertheless, the administration of castor oil twice a day to control rats produced approximately the same result. Although some authors have fallen back upon the nebulous "increased permeability" of the tissues and the bowel wall in hyperthyroidism, it seems reasonable to attribute this effect simply to hyperactivity of intestinal motility as a result of the thyroid hormone.

As pointed out previously, in hypothyroidism the general sluggishness of metabolism produces little wear and tear upon the tissue enzymic constituents and therefore the vitamin requirement is extremely low. A similar situation is found in starvation. On the contrary there is a marked increase in the requirement for certain vitamins in both experimental and clinical hyperthyroidism. In studies of metamorphosis it has been demonstrated that the administration of vitamin A could depress the accelerating effect of thyroxine. Accordingly, in tadpoles (123) and in salamander larvae (130) an antagonism between thyroxine and vitamin A has been suspected, but the evidence is contradictory (273,522). Moreover, in human exophthalmic goiter the values for hepatic stores of vitamin A appear to be normal or even larger than normal. Thus Wolff (534) found an average value of 210 I.U. per g. whereas in control cases succumbing from accidental death the value was only 147 I.U. per g. Similar values reported by Moore (326) were 304 I.U. per g. of liver as against 210 I.U. per g. in the control. Drill

(105) has sifted the mass of contradictory evidence on this subject. The most interesting fact is the difficulty of converting carotene to vitamin A in the absence of thyroid. In clinical hyperthyroidism the serum vitamin A and serum carotene are lower than normal because of an abnormal demand for these substances. The presence of the multiple system of conjugated double bonds in the carotenoids has suggested (408) that these unsaturated molecules deprive thyroxine of its iodine, and so inactivate it.

In the serum a curious paradox is encountered, namely a low concentration of vitamin A in both hypothyroidism and hyperthyroidism. In the first case the lack of vitamin A is due to failure of the conversion of carotene in athyreosis. In the second case, the low value is due to exhaustion of the stores of vitamin A. Godtfredsen (151) has suggested that a decreased hepatic function in hyperthyroidism interferes with vitamin A metabolism. Various experiments have been reported suggesting that vitamin A may impede the action of thyrotropic hormone on the thyroid gland. It is difficult at present to assess the significance of these data. In hyperthyroidism the requirements for B<sub>1</sub>, B<sub>6</sub>, and pantothenic acid are increased (137,109) and the administration of yeast concentrate may help maintain body weight by increasing the intake of food. In the tissues of the hyperthyroid rat the concentration of thiamine is low. Schneider and Burger studied the urinary excretion in hyperthyroid patients (445). Whereas control patients excreted 80 to 100 µg. of thiamine per day, the hyperthyroid individuals excreted 225 µg. daily in a large volume of urine. As regards riboflavin, Kinde (237) reported that hypothyroid rabbits developed pellagra-like lesions; and Greene (159) described similar lesions in two myxedematous patients. In hyperthyroid rats, however, there is no evidence of any preferential requirement of riboflavin (108). In hyperthyroid animals, when the increased demand for Vitamin C is satisfied, the basal metabolic rate declines somewhat. Indeed, the excretion of hyperthyroid patients was below normal (261,115). Whereas a deficiency of C causes hemorrhage in the guinea pig's thyroid, a deficiency of D has no effect upon the gland. Excesses of D, however, increase its output of hormone and produce hyperplasia (175). In the case of Vitamins E and D, the results are so complicated by the iodine content of the food that it is difficult to draw conclusions. Possibly Vitamin E activates the thyroid indirectly through an effect upon the ovary. In hyperthyroid patients the problem arises of "conditioned" vitamin deficiency based on the high metabolic turnover which increases the need for vitamins, (83), and the increased rate of intestinal peristalsis (10). Consequently, the absorption of vitamins may be decreased at a time when the necessity for vitamin is increased. Such disturbances have been described by Strauss (482), Richardson (390), and Salter (410). Castle and his associates (62) cite instances in which disorders of the gastrointestinal

tract so interfere with absorption that the vitamin ingested can not be assimilated, even though the patient will respond satisfactorily to vitamins administered parenterally.

Elsewhere (p. 208) the relation of the thyroid to the anterior lobe of the pituitary has been discussed. The thyroid, however, interacts with all of the other endocrine organs. In some instances this clearly is a peripheral effect, in others it involves tropic hormones mediated through the anterior pituitary. With regard to the posterior pituitary, Mahoney and Sheehan (284) abolished experimental diabetes insipidus in dogs by a total thyroidectomy. The polyuria recurred when thyroid was administered. In the case of the adrenal it is quite clear (206) that large doses of thyroid lead to hypertrophy of the adrenal cortex in young guinea-pigs. There is considerable confusion, however, in the interpretation and application of this fact. For example, Crile (85) believed that there was too much adrenal cortical hormone available in Graves' disease. Conversely, Marine (291) has explained the gross pathogenesis of exophthalmic goiter partly on the basis of underfunctioning of the adrenal cortex or possibly the gonads. At least, it is clear that under the increased metabolic stress of hyperthyroidism the demand for adrenal cortical hormone is greater than in myxedema. Nevertheless, there is very little 17-ketosteroid in the urine of myxedema, and even in Graves' disease there is less than the normal amount. In the case of the pancreas, also, hyperthyroidism increases the need for insulin and conversely total thyroidectomy may lessen it. In myxedema accompanied by diabetes, therefore, the administration of thyroid may intensify the diabetes mellitus while mitigating the hypothyroidism. Similarly Long (274) removed the thyroid in pancreatectomized animals and found that it diminished the ketosis. Recently Houssay (207) has produced a permanent diabetes in animals through the continued use of thyroid. In the case of the parathyroid gland the function seems to be related to the thyroid only through the mediation of the pituitary; this response is probably related to the general increase in metabolic turnover. For example, in the instance of hypoparathyroidism, two distinct increases in calcium mobilization could be demonstrated (22); the first after the administration of parathyroid extract, and the second after the administration of thyroid extract.

Thyroid medication has long been the "old standby" in gynecological endocrine therapy. The enlargement of the thyroid in pregnancy has been recognized for centuries, and during this period of strain simple goiter may develop into a bosselated goiter (200). Similarly in certain cases of dysmenorrhea, hyperplasia of the gland may occur and may disappear subsequently when the uterine disturbance is in remission. In the less severe forms of toxic goiter, patients frequently will improve considerably during recurrent pregnancies. If thyrotoxicosis is severe, however, abortion usually

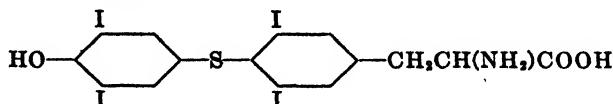
results. It has long been evident that pregnancy increases the demand for iodine and therefore may produce goiter in locations where there is a dearth of that element. Presumably this is related to an increased demand for thyroid hormone. There is considerable evidence of an increased delivery of thyroid hormone into the blood stream during pregnancy, although the data are in need of revision with improved analytical methods (44,253,254; cf. 361). The increased metabolism of pregnancy in rabbits depends upon the presence of the thyroid, and lactation will not occur after complete removal of the thyroid (293). Many years ago Oswald (349) referred to the "exquisite" sensitization of the nervous system in hyperthyroidism. Plummer (366) has described this graphically. Not only is there an increased sensitization but Oswald found that the effect of stimulation of the vagus and depressor nerves was modified markedly after an injection of iodothyroglobulin. In recent years it was shown that vagus stimulation in the continuously hyperthyroid animal tends to accelerate the heart rather than depress it (203). This reversal of the classical response may be of considerable significance in explaining the tachycardia of thyrotoxicosis. Part of the disturbance is due to the simultaneous action of epinephrine. For example, Levy (260) noted an increased effect on blood pressure with epinephrine after stimulation of the nerves of the thyroid gland or with the administration of thyroid extracts. This was prevented by previous removal of the gland. Later such hypersensitivity to epinephrine was suggested as a clinical test for hyperthyroidism (152), but this has not turned out to be very practical. After long continued administration of thyroid to animals, they show greater sensitivity to electrical stimulation (201). This increased sensitivity of the nervous system constitutes a considerable part of the syndrome known clinically as "thyrotoxicosis." Many observers believe that it is due not altogether to an excess of thyroid hormone, but rather to an increased activity of higher sympathetic centers, which in turn is enhanced by the increased sensitivity of the hyperthyroid nervous system (cf. 141).

The clinician or the fundamental scientist frequently assumes that the "toxicity" of hyperthyroidism runs parallel with the degree of the excess of thyroid hormone in the circulation. So far as present evidence is available, this is distinctly not the case. In clinical cases of Graves' disease the patient may appear very exophthalmic and very nervous during times when the basal metabolic rate is nearly normal and the concentration of "hormonal" iodine is within normal limits. The nature of so-called thyroid storm is also complex. It undoubtedly involves the sympathetic nervous system and the adrenal medulla (153). Doubtless the adrenal cortex is also under strain. It is true that such "storms" rarely, if ever, occur in patients whose serum "hormonal" iodine is less than 15  $\mu\text{g.}\%$ , but the presence of storm is

not necessarily correlated with the height of the circulating thyroid hormone. The crisis represents a decompensation in physiological mechanisms which are operating under great strain. Some patients "crack" under less strain than others. The author has seen a patient in storm at 18  $\mu\text{g.}\%$  of serum "hormonal" iodine while not far away another patient with 22  $\mu\text{g.}\%$  was quietly reading his newspaper. The highest value that the author has encountered for serum "hormonal" iodine was 26  $\mu\text{g.}\%$  in a man on the verge of storm. It is suspected that in the treatment of storm, the administration of adrenal cortical extract may be of considerable value. In other words, the exhaustion may be due in part to a relative inadequacy of adrenal cortical function.

### VIII. Thyroid Activity and the Need for Thyroid Hormone

In a previous section it has been pointed out that the activity of the thyroid hormone depends in large measure upon the thyronine nucleus with at least two iodine atoms which are located in the 3,5-positions of the inner aromatic nucleus. From this basic structure certain deviations are possible but in general these have a quantitative rather than a qualitative significance. In other words, diiodothyronine would be the true hormone or "pharmacogen", and the 3',5' iodine atoms "auxopharmacophore" groups, which enhance the physiological effect of the "pharmacogen". It is true that some sort of calorogenic action can be demonstrated in simpler or distorted molecules. For example, Canzanelli and Rapport (58) reported the following sequence of relative activities in dogs: tyrosine 1, diiodotyrosine 7.5, diiodothyronine 1125, and thyroxine 19,125. Likewise in human myxedema diiodotyrosine was 10,000 times less active than thyroxine (495): but a trace of thyroxine arising spontaneously as an impurity conceivably could produce such an effect. When the thyroxine framework is present, the molecule is more likely to produce significant effects. Diiodothyronine is 50 to 75 times less active than thyroxine, but the qualitative effects produced in human myxedema are highly characteristic. Similarly, Means (314, p. 50) states that tetrabromothyronine is 20 times less active than DL-thyroxine and tetrachlorothyroxine between 150 and 200 times. The tetrabromocompound was found to prevent thiouracil goiter in rats, although the "hormonal" iodine fell. Presumably a circulating bromohormone replaced the natural form. Recently, in Means' clinic Lerman (256) has studied a sulfur analogue of thyroxine made by Harington with the following formula:



This thioether was one-fifth as active as thyroxine, but its qualitative effects were typical. As pointed out earlier, some of the peripheral thyroid hormone-blocking agents reported by Woolley (536) and by Winzler (533) show both thyroxine-like action and antithyroxine action.

By and large, the most active substance which has been produced is the levorotatory form of thyroxine (382). Indeed, this substance produces all the qualitative effects of whole thyroid substance when administered to athyreotic mammals. Means (314, 2nd edition, p. 63) reports that the L-isomer is six times as potent as Harington's D-isomer when tested in human myxedema. This positive finding is supported by studies in animals by Foster, Palmer and Leland (134) and Reineke and Turner (382). These investigators found that L-thyroxine was twice as active as the DL-mixture. Although they concluded that the D-isomer was inert (by calculation), probably the data are not accurate enough to detect the lower activity of the D-form.

The substance which is next highest in potency appears to be diiodothyronine (186). The polypeptide of thyroxine (186,425) has a potency which is very close to that of L-thyroxine, when calculated in terms of its active prosthetic group, or in terms of iodine.

It has been disputed for a long time whether the content of L-thyroxine in whole thyroid or thyroglobulin is the best index of the activity of any given thyroid preparation. In myxedematous patients the respective responses to natural thyroxine polypeptide and to whole thyroid are identical when administered in equi-iodine dosage. Moreover, the myxedematous patient responds much more vigorously to the whole thyroid material than to the thyroxine which it contains (319). On the other hand, in intact normal guinea pigs (134) the responses evoked by whole thyroid are very similar to those of the equivalent L-thyroxine, provided the doses are assigned on the basis of thyroxine content. This problem still remains unsettled. Recent work seems to indicate (382) that D-thyroxine itself has relatively little activity even in human myxedema (256), although several such cases have responded to supposedly pure D-thyroxine. Therefore, one must assume that the high effectiveness of thyroglobulin is due both to levorotation and to some prolonging or enhancing effect of the polypeptide chain (233). Harington (187) suggested that diiodotyrosine when linked with thyroxine and other amino acids may "form the true active principle of the gland."

Apparently the diiodotyrosine moiety in thyroglobulin has no endocrine potency in itself. Hydrolyzates from which the "T" fraction has been separated are inactive (257). Furthermore, in iodinated protein preparations, as already pointed out (335), the characteristic hormonal activity does not arise until all the tyrosine groups in the protein have been saturated with iodine. Thereafter activity is produced very rapidly, presum-

ably by an oxidation-reduction process. This step-wise action of elementary iodine is illustrated in Fig. 5. In Fig. 14 are shown the corresponding "T" and "D" fractions of the blood serum proteins.

Another controversy has arisen because of the poor absorption of thyroxine crystals. If pure, dry thyroxine is administered to man, a large percentage may fail to be absorbed, but from the material dissolved in dilute alkali and ingested in a large volume of water, the absorption may be as high as 80% (499,500), or 100% in rats (324).

Nomenclature for Serum Iodine Fractions

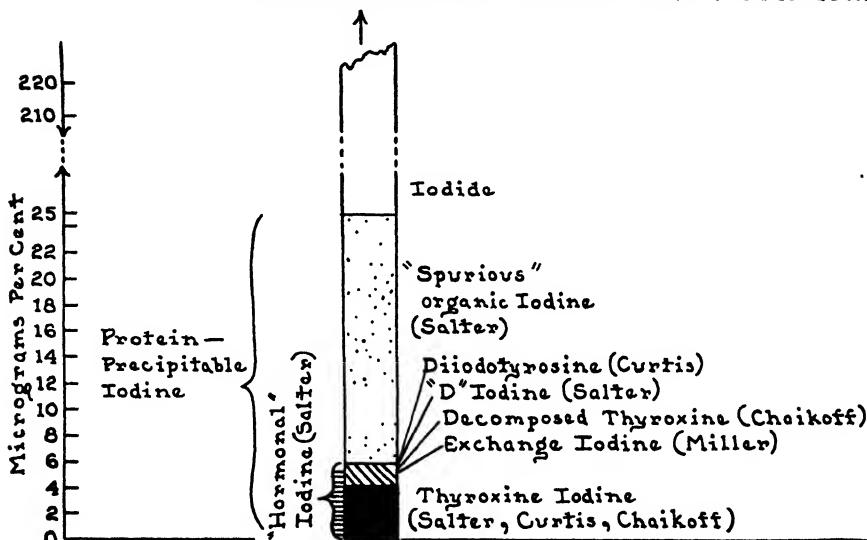
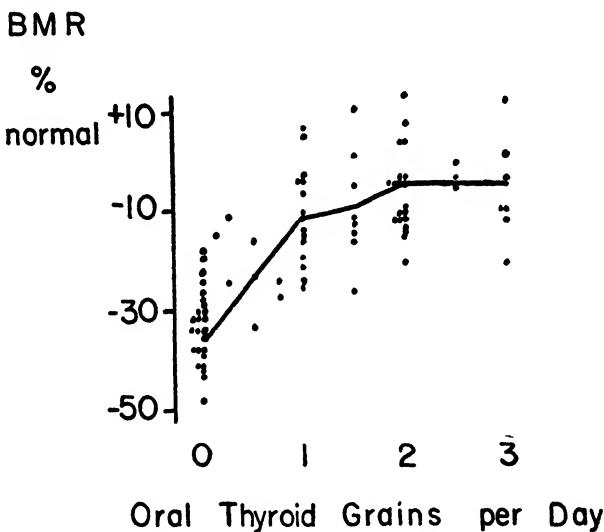


FIG. 14.—After hydrolysis, microchemical analysis of the protein-bound iodine in blood serum reveals both a thyroxine-like and diiodotyrosine-like fraction. It is still disputed whether the latter is an artifact, arising in the course of chemical manipulation. The figure also shows the "spurious" elevation occurring under heavy iodide therapy, which is not related to increased thyroid function.

The problem of how much hormone is produced each day by the gland under natural circumstances has received a rather definitive answer by studies (317) of the effect of medication in myxedematous patients. Such patients afford a very good test object for the assay of the drug (530). As shown in Fig. 15 there is a statistical correlation between the basal metabolic rate and the oral dosage of thyroid in 28 cases of hypothyroidism.

Magnus-Levy (283) showed that in the cretin after the stopping of thyroid therapy, the organism's BMR declined in inverse logarithmic fashion. In other words, each day the organism used a constant percentage of its remaining store of thyroid hormone. This depot, of course, would be

only that stored in the general tissues at large, because the gland was no longer available as a source of hormone. A study of the requirement of



#### STATISTICAL RELATIONSHIP BETWEEN BMR

FIG. 15.—Statistical relationship between BMR and dosage of oral thyroid in twenty-eight cases. The heavy line connects the average values for the BMR at each level of thyroid dosage. (From Winkler, Criscuolo, and Lavietes, *J. Clin. Invest.* **22**, 533, 1943.)

TABLE VII  
RESPONSE TO THYROXINE AND DESICCATED THYROID\*

Medication	Iodine in Substance Used (mg. per daily dose)		Level of BMR
	Thyroxine- iodine	Total iodine	
None.....	0	0	-15
Desiccated thyroid.....	0.17	0.6	0
$C_{18}H_{11}O_4NI_4$ .....	0.5	0.5	+19
Desiccated thyroid.....	1.4	4.7	+30
$C_{18}H_{11}O_4NI_4$ .....	4.7	4.7	+60

\* From Salter, *The Endocrine Function of Iodine*. Harvard Univ. Press, Cambridge, Mass., 1940, p. 109.

thyroid and thyroxine at several levels of metabolism in a woman after thyroidectomy, is given in Table VII (258). The decay curve found in such individuals was reproduced approximately by the decline of metabolism under iodide medication during a hyperthyroid phase of the patient's stay

in the hospital. It appears that by integrating the decay curve in such patients it is possible to arrive at an estimate of the stored hormone in the

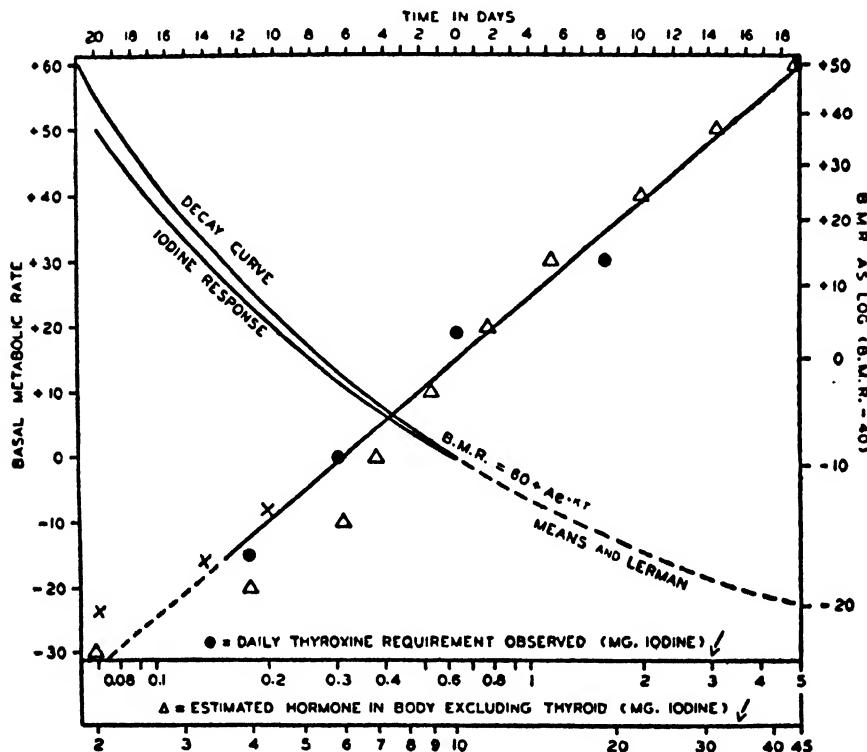


FIG. 16.—The daily requirement of thyroid hormone varies with the metabolic rate. The figure collates data of Means and Lerman and of Salter and Lerman, supplemented by data (crosses) of Thompson. When the appropriate scales are used, the straight line serves three purposes: (1) with the topmost abscissa and the right-hand ordinate, the decay curve is illustrated; (2) with the left-hand ordinate and the lowest abscissa (open triangles), the estimated hormone stored in tissues (exclusive of the thyroid) is shown; (3) with the intermediate abscissa (solid circles) and the left-hand ordinate are shown the requirement of thyroxine (in terms of iodine) at several levels of thyroid activity. These data are continuous with those of Thompson (crosses) for myxedema. (From Salter, *Physiol. Revs.* 20, 365, 1940.)

body tissues. Moreover, this store is proportional to the daily dosage of thyroid required at any given level of caloric turnover.

Data on the thyroxine requirements at various BMR levels are combined in Fig. 16 (47,413,497). They show the basal metabolic rate attained is a logarithmic function of the dosage administered. Of course, in conducting such observations it is necessary to be sure that a steady state is reached at

each dosage. There is some deviation from a straight line which may reflect the fact that in the course of the observations the organism changes structurally: so that in effect one cannot carry out the whole span of observations on a single creature. By integrating such observations against time it is possible to deduce the total amount of hormone stored in the body at any given level of basal metabolic rate. It is equivalent to at least 10 mg. of thyroxine.

Iodide, itself, can probably alter the activity of the thyroid gland directly. This is a complex problem which has confused many investigators because it is triphasic. (i) At very low levels of iodide intake (sufficiently low to produce hyperplasia through pituitary action), a trace of iodide will allay the hyperplastic response. (ii) A higher iodine intake spurs the gland on to increased activity and output of hormone (271). (iii) At very high intakes of iodide, the gland "grows lazy" (as it were) and involutes; presumably because its daily quota of hormone is readily synthesized.

A further problem is the effect on circulating hormone of the thyroid gland, if present. The gland can store some of the excess hormone in the follicles, or it can inactivate it. In this way the gland may be said to act as a sort of "buffer", protecting the organism against the too enthusiastic therapy of the physician. A third problem, finally, is the reaction of the tissue cells themselves.

It is interesting that the total storage of "hormonal" iodine in the tissues of euthyroid individuals, as computed, turns out to be somewhat more than 10 mg. This value is not very far from that calculated from the summatd iodine content of various organs in the body (486,412). It is instructive, also, that the curve so calculated can be used to predict the rate of decay of the basal metabolic rate in a patient whose thyroid gland has been removed. In general, then, the metabolic rate is a crudely logarithmic function of the daily supply of hormone. Furthermore, the daily thyroxine requirement for the maintenance of metabolic equilibrium is proportional to the estimated hormone stored in body tissues, exclusive of the thyroid. Incidentally, the BMR is satisfactorily predictable also from the concentration of plasma-precipitable iodine plotted in the same fashion as the other functions just described (414,276). This fact is illustrated by Fig. 16. The combined data afford a very satisfactory quantitative summary, then, of the effect of the thyroid in the human body. Indeed, in the case of no other hormone have we so striking a description of endocrine effect in quantitative terms. These data afford also a quantitative justification for using the concentration of circulating "hormonal iodine," recorded in a steady state, as a measure of thyroid activity in the human organism.

As regards the amount of thyroid consumed, it appears that normally the human gland must supply the body tissues with more than a fifth of a mg. of thyroxine daily. Under practical conditions, the physician must

administer a larger amount, because single large doses are not efficiently utilized. In terms of U.S.P. thyroid this amounts to at least 3 grains (195 mg.) daily, possibly containing 0.2 mg. of thyroxine. In profound myxedema almost no hormone is available, while with a metabolic rate of plus 50 or 60, the daily thyroxine requirement may be a matter of 4 or 5 mg. This last amount would produce brisk hyperthyroidism and is not recommended.

In recent years the suggestion has been made (69,325) that thyroid hormone may arise in body tissues other than the thyroid. The amounts actually produced, however, must be very small at best.

In subjects without myxedema, the tolerance to oral thyroid and the reaction to intravenous thyroxine is much more variable than in the truly myxedematous organism (531). It seems fair to say that the individual possessing an active thyroid has a definite protection against an excess of thyroid and therefore a very marked "tolerance". The best explanation seems to be that the non-myxedematous subject is able to inactivate thyroid substance (and intravenous thyroxine) whereas the myxedematous individual must depend upon slower processes of degradation and excretion.

In Fig. 17 is shown the response in the basal metabolic rates of six non-myxedematous subjects to various dosages of thyroxine. For comparison the figure gives the usual response of myxedematous subjects. The more sluggish reaction of the normal individual is striking.

The use of an isotope has the double advantage of tagging a given sample of iodide in point of time and of being measurable in the intact human subject. Using radioiodide, several authors (227,172,173,193) agree that the fate of ingested iodide is determined largely by two factors, i.e., (a) thyroid function and (b) renal function. Ordinarily only about 2% of a small dose of tracer iodide appears in the feces. Hypothyroid individuals excrete over 85% in the urine in 5 days as compared with some 65% in normal subjects in 2 days, and less for hyperthyroid individuals. These values represent tracer- or test-dosage effects, i.e., when the total iodide given orally to a human adult is under 99  $\mu$ g. In short, they reflect the natural state of iodide metabolism in man.

The appearance and disappearance of tracer iodide in the thyroid affords a useful means of appraising the activity of the gland. Time curves observed by several investigators (172,173,478,64) are in essential agreement, and are inversely correlated with curves of urinary excretion. The radioactivity of the neck observed in myxedema is only twice or thrice that of the thigh. In euthyroidism, the thyroid continues to accumulate tracer for 24 to 48 hours, until some 20% of the tracer is stored in it. In hyperthyroidism the tracer is accumulated faster and to a larger degree, but is soon lost, as the

hyperactive gland expends its synthesized hormone. Usually in 6 to 12 hours about 45% of the tracer, as a maximum, is found in the neck. In actual fact, these data do not measure thyroid function but rather a degree of thyroplasia. Indeed, even in the presence of myxedema, patients suffering from struma lymphomatosa or from cyanate goiter may show a marked and rapid accumulation of tracer. Usually, however, thyroplasia is a good index of the synthesis and storage of thyroid hormone.

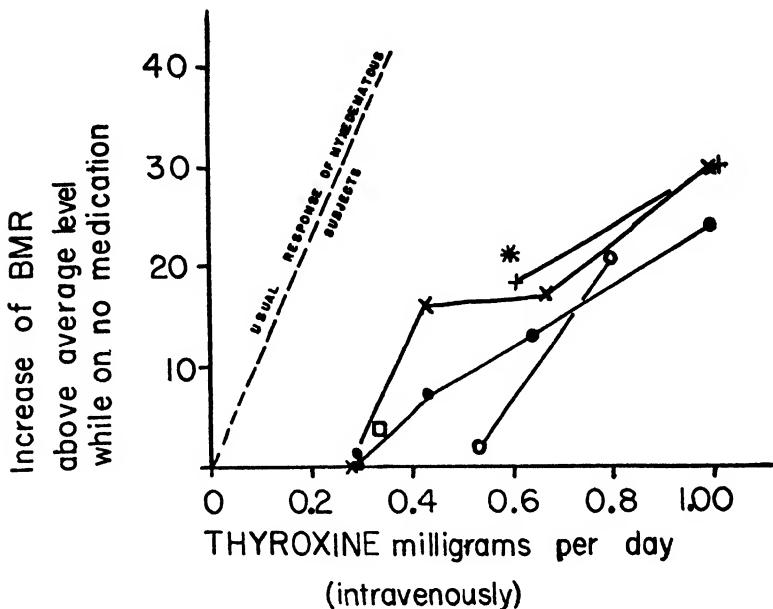


FIG. 17.—Response of BMR in six non-myxedematous subjects to various doses of thyroxine. Each point corresponds to the average BMR after a course of two weeks or more of intravenous thyroxine in the dose indicated. There is no response unless more than 0.3 mg. of thyroxine are given daily, and even with larger doses, the response is much less than that usually found in myxedematous subjects. (From Winkler, Lavietes, Robbins, and Man, *J. Clin. Invest.* **22**, 541, 1943.)

When doses over 10 mg. of sodium iodide are ingested by a normal or hyperthyroid human adult or an animal, the percentage of tracer trapped by the thyroid rises rapidly to a peak and then declines exponentially, much as does the iodide in the circulating blood (172,227,479,514,494). This rapid accumulation represents the temporary fixation of iodide which may be reversed rapidly by the ingestion of one gm. of potassium thiocyanate.

The amount of tracer iodide trapped by the thyroid depends on the disposition of iodide by the kidneys and other tissues. When appropriate

corrections are made for such factors (227), the accumulation rate and thyroidal iodide clearance can be used to measure thyroid function. Thus in exophthalmic goiter the mean accumulation rate is over 8 times the rate for euthyroid individuals with thyroid tumors. More reliable is the thyroidal iodide clearance, defined as the volume of plasma cleared of its iodide content by the thyroid per minute. Representative values for euthyroid subjects lie between 2 and 13 ml. per minute, as compared with values for hyperthyroid patients between 20 and 228 ml. per minute. Thus, in round numbers the "average" normal thyroid clears 10 ml. per minute; whereas the "average" exophthalmic goiter clears 130. The clearance is a sensitive index, because the maximal accumulation of tracer in Graves' disease rarely exceeds thrice the euthyroid value. These mean values, of course, are only approximate; they are based on an assumed volume of distribution of 350 ml. per kg. and an accumulation rate of 215 ml. per hour for euthyroid subjects.

In applying tracer iodide to study the turnover rates of iodine or of thyroid hormone, it is not easy to interpret the data expressed in "counts per minute" of radioactivity unless the concentration of stable iodine,  $I^{127}$ , is also known for each chemical individual involved. Thus in the blood serum of the normal rat, the concentration of "hormonal" iodine (equivalent to thyroxine) is approximately 2.5  $\mu\text{g}$ . per 100 ml. After the injection of a single dose of tracer iodide (without appreciable carrier), this tracer is uniformly distributed throughout the body fluids. From this reserve the new "hormonal" iodine is drawn by synthesis. General trends complicate the interpretation of the data (420). First, a suitable correction must be made for the progressive physical decay of the isotope. Secondly, a metabolic dilution of the labeled iodide reserve occurs, as iodide is excreted and fresh unlabeled iodide replaces it. Thirdly, as the labeled "hormonal" iodine is formed, it mingles with unlabeled hormone previously in existence. Fourthly, continuous degradation of the "hormonal" iodine occurs as it engages in tissue metabolism. Thus, at any time,  $t_0$ , after the injection of the tracer, the concentration of newly made hormone,  $H$ , which has been synthesized must be estimated from the equation

$$\frac{dH}{dt} \left(1 - \frac{L}{2.5}\right) = \frac{dL}{dt}$$

where  $L$  indicates the concentration of labeled "hormonal" iodine in the blood or tissue. Hence, at the time,  $t_0$ , the amount of newly made hormone can be calculated from the equation

$$H_{t_0} = -2.5 \ln \left(1 - \frac{L_{t_0}}{2.5}\right)$$

The evaluation of  $L_{t_0}$  is complicated by the fact that the specific radioactivity,  $\sigma$ , of the iodide reserve declines continuously according to the equation

$$\sigma = \frac{d\sigma}{-K dt} \quad \text{or} \quad \frac{-d \log \sigma}{dt} = K$$

It is necessary, therefore, to integrate two variables which interact progressively with time, i.e.,

$$\frac{dL}{dt} = \frac{dA}{\sigma dt} = \frac{1}{K_0} e^{k_1 t} \frac{dA}{dt}$$

An example will illustrate the method. Two series of rats were studied. In Series A the "hormonal" iodine was continuously 2.5  $\mu\text{g.}\%$  and the inorganic iodine 0.5  $\mu\text{g.}\%$ . Series B was treated for 6 months with thiouracil until larger goiters were present and the "hormonal" iodine was only 0.5  $\mu\text{g.}\%$ . Thus Series A mirrors normal thyroid activity and Series B the reawakening of thyroid function after months of blockage. In Series A, the concentration of labeled "hormonal" iodine accumulated in 48 hours can be calculated as follows:

$$L_{t_0} = \frac{-4}{k_0} \int_0^{48} \frac{e^{k_1 t}}{t^2} dt$$

Thus  $L_{t_0}$  was found to be 0.9  $\mu\text{g.}\%$ ; and from this value it could be computed that the whole 200 gram rat metabolized about one  $\mu\text{g.}$  of thyroxine (or its equivalent) per day (421). In series B the restoration of organic iodine in muscle was delayed by several days because the thyroid gland did not immediately recover from the pre-existing poisoning with thiouracil.

When athyreotic patients were studied before and after therapy, the following changes were noted by the use of tracer iodide (227). The renal excretion increased in rate; the disappearance rate of the plasma increased by half; the extrarenal disposal rate (in miscellaneous tissues) remained unchanged; the renal clearance increased; the thyroidal clearance and accumulation rate became nil; and the approximate volume of distribution decreased by 4 l. All of the observations could have been anticipated from the findings previously described.

If iodine-containing materials are being tested, special preliminary knowledge must be available concerning the metabolic behavior of these compounds. If, for example, they linger in the blood and thus yield high concentrations of iodine, the analysis for plasma-precipitable iodine may not reflect true "hormonal" iodine. After the administration of radio-opaque substances in huge doses, the overwhelming accumulation of iodide and

of organically bound iodine may "swamp" the analytical procedures now available for "hormonal" iodine. For example, both Salter (416) and Karandikar (224) have called attention to the "spurious" elevation of precipitable iodine-containing material in blood plasma when patients are exposed to organic medicaments containing a high percentage of iodine. Among such organic iodine compounds are the sodium salt of monoiodomethanesulfonic acid ("Skiodan"); radio-opaque preparations like phenyldiiodohydroxyphenylpropionic acid ("Priodax"); and iodized oils. On the other hand, in experienced hands, the effectiveness of small daily doses of routine thyroid medication (as described in the U.S.P.) can safely be followed by analysis of serum, with due allowance for a lag in the response. It is not easy to procure and maintain many human myxedematous individuals under such steady conditions and therefore for many purposes it is necessary to use animal material. To this end, therefore, a number of procedures have been adapted for the quantitative measurement of thyroid activity. It must suffice here to survey briefly some of the more useful of these methods.

#### A. OXYGEN CONSUMPTION

For decades the measurement of the oxygen consumption of animals under standard conditions has been utilized as an indirect measure of the activity of thyroid preparations or of thyroxine. The most satisfactory assay of a thyroid preparation is that made in human cases of myxedema under well controlled conditions in a special study ward of a large clinic (414). Such a test has the advantage that it affords an unmistakable qualitative control of the material concomitantly with a quantitative measurement of activity in terms of total calories. Ordinarily the indirect method of calorimetry based on oxygen consumption, and interpreted by the standards of Du Bois (111) is adequate. In general, it is best to attempt to approximate the response given by a known amount of thyroxine e.g., 0.5 mg. For use in animals there are two general types of apparatus, the open-circuit apparatus (60) and the closed type of apparatus (388). Another useful apparatus is that of Lee (251) for use in rats.

#### B. METAMORPHOSIS

In 1912, Gudernatsch (162) demonstrated that the gland which affected most actively the metamorphosis of the tadpole was the thyroid. Since that time, this phenomenon has been used in a quantitative fashion to gauge the activity of unknown preparations. For example, with axolotl, Uhlenhuth (512) showed that as little as 0.03  $\mu$ g. of thyroid iodine in a liter of water would cause metamorphosis within two weeks. By contrast, between 33 and 86 times this amount of iodide had no effect. In Romeis's

experiments (405) the tadpoles were left in 500 cc. of a solution of thyroxine, 1 to one million or 1 to ten million, for a period of 24 to 48 hours, and then maintained in 100 cc. of water. After two such treatments in the thyroxine solution metamorphosis became apparent. These tests on amphibian metamorphosis have the advantage that very small amounts of material can be used (143,543). They have the disadvantage of lack of specificity because large amounts of iodide and other iodine-containing substances can force metamorphosis, particularly if the animals possess

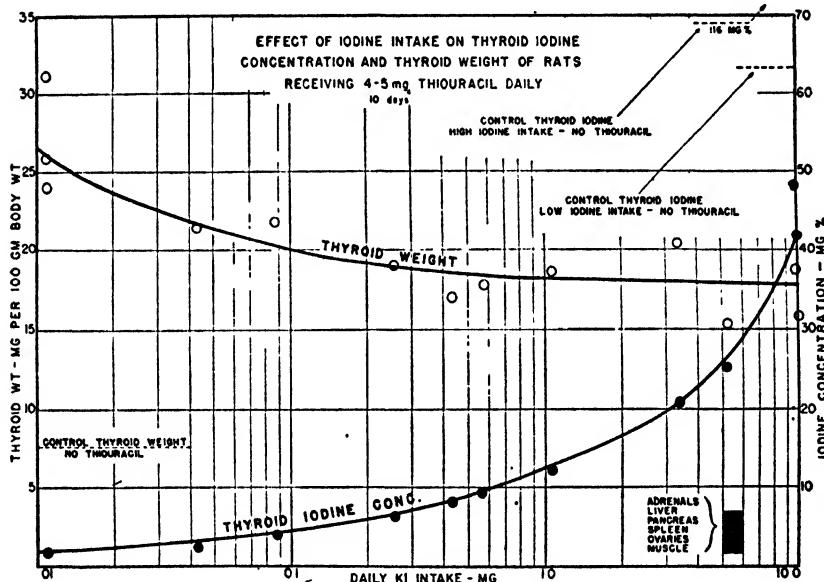


FIG. 18.—As a measure of glandular activity both the thyroid weight and the thyroid iodine concentration may be used.

an intact pituitary and thyroid. A comprehensive discussion of this method is given by Kendall (232).

### C. ANTIGOITROGENIC ACTIVITY

The goitrogenic effect of compounds like thiouracil can be combatted by the simultaneous administration of thyroxine. Astwood and Bissell (21) pointed out that at various dosages of these goitrogens, one could find an equivalent amount of thyroxine which would prevent any effect on the thyroid. This method has been used to gauge the potency of thyroid material in standard animals, e.g., rats (450). McGinty (310) has studied in rats the relation between iodine utilization and glandular activity, when the animals are exposed to goitrogens. Such data are summarized in Fig. 18.

#### D. PLUMAGE TESTS

It has long been known that the endocrine system affected the plumage of fowl and birds. In 1925, Torrey and Horning studied the effects of thyroid feeding on the moulting process and feather structure of the domestic fowl (504). The feeding of iodide in control experiments did not affect the plumage, but the administration of thyroid material tended to produce hen-feathering in the Rhode Island Red male. Later, chickens were found to molt in 7 or 10 days after the administration of suitable amounts of the material (541). The new feathers were generally white or pale. It was possible to compare various thyroid preparations by this criterion. For example, 10 mg. of thyroxine produced molting and all of the morphogenetic changes caused by 5 to 10 g. of desiccated thyroid. By the intracutaneous injections of known amounts of thyroid material, extremely small dosages can be used (90). The effect is read locally, near the site of injection.

#### IX. Iodine Metabolism and the Thyroid Gland

In the foregoing sections it has been noted that the thyroid gland serves several functions with respect to iodine. (1) It serves as a reservoir for thyroid hormone, which it fixes in the form of thyroglobulin and stores within its follicles. (2) It regulates the release of this stored hormone, under the continuous "tonic" control of the pituitary gland. (3) It traps with considerable efficiency the breakdown products of metabolism, thus conserving iodine during periods when the iodine supply from exogenous sources is poor. (4) If an excess of thyroid hormone is introduced into the body, the gland is able to remove an excess of this drug, by destroying it or storing it. In this way, despite wide fluctuations in the supply of iodine and in the demand for hormone, the gland is able to maintain a fairly uniform concentration of thyroid hormone in the circulating plasma. Much of this delicate control centers in the hypothalamus and in the pituitary itself. Nevertheless, even in the absence of the pituitary there seems to be a sort of basal, sluggish action of the thyroid which is primitive and operates on its own initiative (188).

In general, the normal adult tissues at large probably receive each day about 200  $\mu$ g. of iodine in the form of thyroxine or some derivative thereof, though this amount is probably more than necessary. It would be difficult to prove that a normal adult receiving only 40  $\mu$ g. of iodine a day was necessarily headed for a deficiency state.

Furthermore, growing babies need from about 22 to 44  $\mu$ g. daily and older children a little more during the activity of growth. During pregnancy and lactation the requirement increases considerably (114). Very strenuous exercise, and stresses and strains such as fever or infection, increase the demand.

With other organic compounds containing iodine, notably the sodium salt of monoiodomethanesulfonic acid ("Skiodan") and beta-(4-hydroxy-3,5-diiodophenyl)-alpha-phenylpropionic acid ("Priodax"), the total iodine

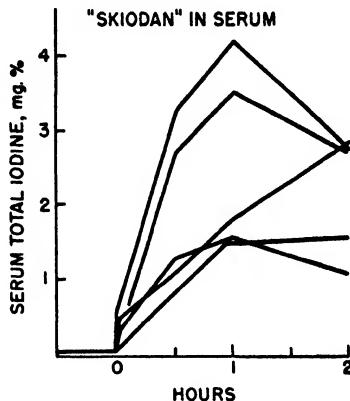


FIG. 19.—Total iodine in human blood serum after intrathecal instillation of sodium salt of monoiodomethane sulfonic acid. (From Salter, Metabolic circuit of the thyroid hormone, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 364, 1949.)

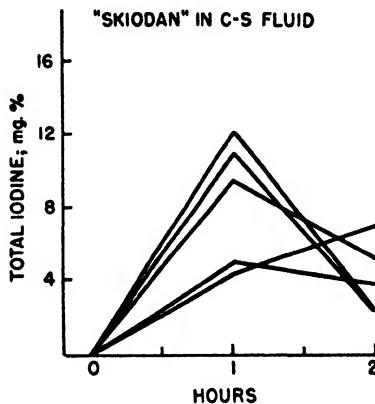


FIG. 20.—Total iodine in human cerebrospinal fluid after intrathecal instillation of sodium salt of monoiodomethane sulfonic acid. (From Salter, Metabolic circuit of the thyroid hormone, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 363, 1949.)

in the blood increases, even though the "Skiodan" be instilled into the spinal canal and the "Priodax" injected intraperitoneally (432). In Figs. 19 and 20 are shown the rise of iodine concentration in body fluids following the administration of "Skiodan." The serum concentration reaches a

maximum about 90 minutes after the instillation of the drug (not a usual therapeutic procedure!); and at this time half of the material is still in organic combination. Likewise, Keating, and Albert (227) have studied the fate of radioactive diiodotyrosine, iodocasein, and thyroxine, as will be described presently. When not held back by a large mass of digesting food (411), salts like sodium iodide are rapidly absorbed (166,227); in the absence of severe myxedema, absorption is finished in less than two hours. As it is absorbed, the iodide appears in the circulating blood. Serial observations with tagged iodide by McConahey and associates (308) have revealed two successive trends in serum iodide concentration. In the first two hours there is a rapid rise (due to absorption) followed by a rapid fall (due to distribution in various body fluids). Later, there occurs a prolonged fall, which reflects the combined functions of the thyroid and kidneys. In hypothyroid individuals this lasts as much as 12 to 24 hours; in euthyroid subjects 6 to 12 hours; and in hyperthyroid individuals often less than 6 hours.

Ordinarily very little iodine is lost through the breath, although amounts as high as 10  $\mu\text{g}$ . in 24 hours have been recorded (119). Karandikar (224) found that after a 14 mc. dose of  $I^{131}$  given orally, a patient who had excreted over 93% of the dose in 48 hours, still showed almost two microcuries of specific activity in each expiration, i.e., (72000 c.p.m.). Inasmuch as the total residual activity in the body at the time was less than a millicurie (960 microcuries), and the serum indicated 250 c.p.m./cc., it is to be concluded that the respiratory excretion may provide an interesting excretory channel to study. Probably the radioactivity of the expired air reflects directly the radioactivity of the blood serum. On low intakes, e.g., 17  $\mu\text{g}$ ., about 84% of the iodine is in the urine and less than one-quarter in the feces (439,227). If iodide is ingested in excess, the urinary secretion increases accordingly. On the lower intakes, severe sweating may divert the urinary iodine and so reduce the urinary excretion almost to nothing (489). In Fig. 21 are shown measurements of radioiodide in human urine by means of radioactive iodide. Normal men excrete about 6% of the body's iodide reserve each hour (227). The renal clearance amounts to some 38 ml. per minute, and varies but little at various plasma concentrations of iodide. This suggests that ordinarily iodide is passively absorbed by the renal tubules.

Fecal excretion may be increased by severe diarrhea or by a very large intake of iodide, such as may occur in medication for tertiary syphilis. Obviously urinary excretion and fecal excretion tend to bear a reciprocal relationship to each other; but usually urinary excretion predominates. For example, on an intake of 155  $\mu\text{g}$ . of iodide a day, the urine contained 116  $\mu\text{g}$ . or 75% of the total, the sweat 24  $\mu\text{g}$ . and the feces only 5  $\mu\text{g}$ . (439).

Keating and Albert (227) found that only 2% of iodine ingested in the form of diiodotyrosine, iodocaseine and thyroxine appeared in the feces. In other words, less than 10% of the ingested iodine failed to be excreted.

The rate of absorption of iodide is an exponential function of the amount in the digestive tract, about 5% being absorbed per minute into the blood and body fluids (227). It disappears from the blood of normal individuals at the rate of about 9.5% each hour. The concentration in the blood at a given moment determines the rate of trapping by the thyroid, the excretion in the urine and its utilization by various peripheral tissues. In all organs

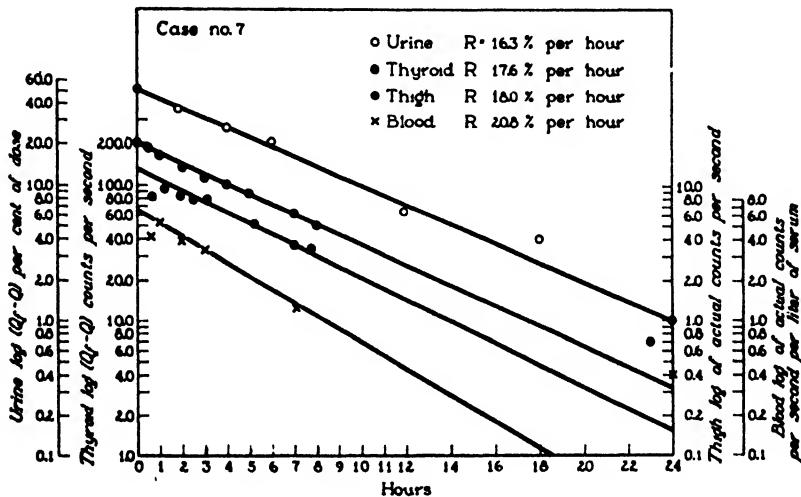


FIG. 21.—Curves of urinary excretion, thyroidal accumulation, radioactivity in the blood and radioactivity in the thigh plotted on a semilogarithmic scale. The significance of the relationship illustrated by the figures is discussed in the text; it is seen that all four curves have approximately the same rate constant. The methods involved in plotting and calculating these values are discussed in the text. (From Keating and Albert, *Recent Progress in Hormone Res.* 4, 429, 1949.)

except the thyroid, the content of iodide may be explained in terms of uniform distribution throughout extracellular body water. In the normal thyroid gland about 2.5% of inorganic iodide accumulates each hour, as shown in Fig. 22. This rate corresponds to a thyroidal iodide clearance of about 10 ml. per minute. Corresponding values in hyperthyroidism rise to 20% trapped each hour or 130 ml. per minute cleared. The miscellaneous tissues, exclusive of kidney and thyroid, utilize iodide and dispose of it at rates of only 1 to 2% each hour. Indeed their total activity account for only about 12% of the total iodide reserve.

When ingested, organic combinations of iodine are absorbed more slowly than is iodide. Moreover, the fecal loss may vary from 11 to 60% of the

ingested iodine (227). In hypothyroid subjects the rate of absorption is slower than for normal people; but in hyperthyroidism increased intestinal activity may also limit the absorption. Soon after ingestion a significant part of ingested organic iodine (e.g., in iodocasein or thyroglobulin) may be converted to iodide. The liver tends to store temporarily organic iodine compounds; and part of this accumulation is excreted in the bile, largely in a deiodinated (inorganic) state. When ingested in soluble form some

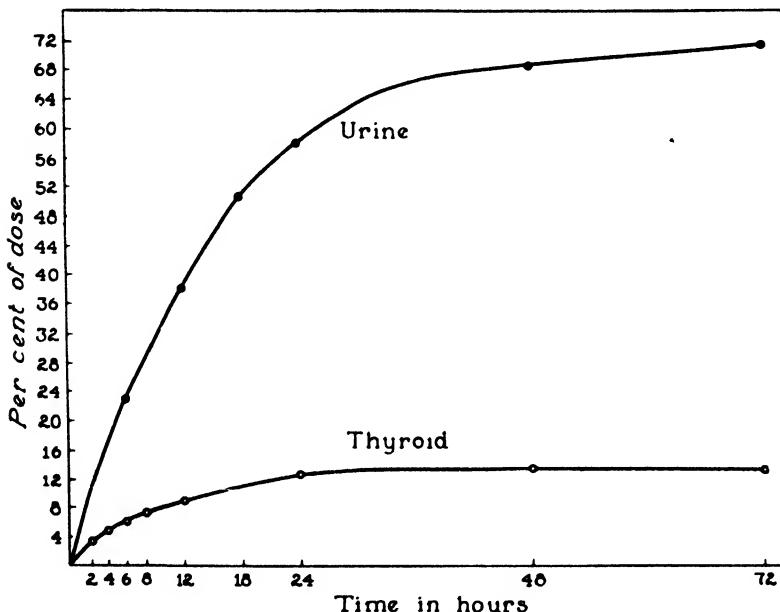


FIG. 22.—Comparison of urinary excretion and thyroidal accumulation in a euthyroid patient with thyroid tumor. Here most of the dose goes to the urine and only 15% to the thyroid. Nonetheless both curves level off at approximately the same time (48 hours). Subsequent secretion by the thyroid and constant excretion by the kidneys occur here. (From Keating and Albert, *Recent Progress in Hormone Res.* 4, 429, 1949.)

organic compounds, e.g., thyroxine in solution, may be efficiently absorbed without de-iodination; but progressive conversion to iodide occurs after assimilation. In general, therefore, the ingestion of most organic compounds of iodine leads to the appearance in the blood of both an elevated iodide and an elevated organic iodine concentration. Iodine may also reach the blood stream from medication administered parenterally, e.g., in the spinal canal. Such iodine may yield "spurious" values for serum "hormonal" iodine.

While the major part of the iodine ingested in organic combination ends up as urinary iodide, organically bound iodine may also appear in the

urine. For thyroglobulin, iodinated casein and thyroxine this moiety may amount to 5 or even 15% of the total urinary iodine, and probably represents diiodotyrosine which has escaped conversion to iodide. This organic fraction becomes relatively less significant as time passes, so that after the fourth day it is trivial. Even after the intravenous injection of thyroxine, Elmer (117) found no thyroxine in the urine.

Preparations of iodinated casein, as described by Reineke and Turner (382,384,508) are effective in human myxedema at a daily oral dosage of 60 to 70 mg. The fate of such compounds is best studied not merely in the myxedematous state but also after the patient's metamorphosis to euthyroidism. Keating and Albert (227) calibrated their subject with tracer iodide both before and after relief of the hypothyroidism. The initial observations of the plasma tracer showed that even after 48 hours only a suspicion of protein-bound tracer could be detected, although this amounted to 52% of the very low protein-precipitable iodine. The tracer iodide rapidly disappeared from peripheral tissues. The final recoveries of the tracer iodide amounted to 2% in the feces and 94% in the urine. Then to this calibrated individual 70 mg. of radioactive iodocasein was administered. Within an hour, half of the tracer in the blood plasma was protein-bound. The ultimate fecal excretion was 59% and the urinary excretion 44%. Ultimately the plasma precipitable iodine rose from its early value of 1  $\mu\text{g.}\%$  up to 12. As the patient became euthyroid, she was calibrated again with tracer iodide. Again 2% of the tracer appeared in the feces and 97% in the urine. No protein-bound tracer was detected in the plasma. The only change was an increased rate of disposal, as expected. At this stage the administration of iodocasein had caused the protein-precipitable iodine to be doubled.

In a similar experiment with thyroglobulin, at the end of an hour the increased plasma iodine was one-third in iodide form. The ultimate excretions of iodine (preponderantly iodide) were, respectively, 60% urinary and 11% fecal. This was true both before and after relief of the hypothyroidism.

The *dl* form of radioactive thyroxine was administered by Keating and Albert (227) to a hypothyroid patient who probably had a small residual thyroid function. Indeed about 10% of the calibrating dose of 100 microcuries of tracer was fixed in the neck. By the third day of the calibration period (at a dosage of 0.5 mg. thyroxine daily) practically all of the tracer appeared in the plasma-precipitable fraction. Later, however, when the patient had become euthyroid, the tracer was all in the form of iodide, and no cervical fixation of the calibrating iodide tracer was observed. Urinary excretion was 69% before treatment and 78% after treatment. With respect to the fate of the radiothyroxine ingested, none was fixed in the neck. Soon after ingestion, the protein-bound iodine of the plasma increased. Only much later was a small iodide fraction detected. The respective renal

excretions (before and after therapy) were 35 and 55% of the dose; the corresponding fecal excretions 39 and 23%. For over a day the renal excretion was linear with respect to time.

### X. Iodine Metabolism in Thyroid Dysfunction

Endemic goiter ordinarily occurs in regions of iodine paucity. Therefore, as might be expected, in this condition iodine excretion tends to be normal or slightly subnormal. When the thyroid is removed completely, the urinary excretion is more than doubled for two or three weeks while the tissue stores of iodine are declining to the hypothyroid level (485). Thereafter, the iodine excretion again becomes normal, because it is dependent simply upon ingested iodine. When a large dose of iodide is administered to such a thyroidectomized animal, the blood iodide level rises faster than normally because there is no thyroid buffer to soak up the medication (81). Similarly urinary excretion appears more promptly and is more pronounced. These findings simply show that without a thyroid gland the organism stores iodine poorly. By way of contrast, when some form of thyroid hormone is administered to patients or animals with myxedema, much of the extra iodine appears in the urine and feces. Therefore, without the thyroid, the urinary excretion of iodine predominates, in contrast to findings in the normal and hyperthyroid states.

In the hyperthyroid patient the iodine balance tends to become negative and in severe cases the deficit may exceed 200  $\mu\text{g}$ . a day. Several factors contribute to this negative iodine balance; excessive sweating, increased intestinal activity, and increased excretion of thyroid hormone through the bile. Indeed, after a rather large intravenous dose of thyroxine, the bile may even contain active thyroid hormone (26), although this must represent a rather unusual situation.

The excretion of iodine depends upon the function of the thyroid rather than its morphologic character. For example, in nontoxic nodular goiter, the excretion of iodine tends to be normal; whereas, if such a nodular goiter becomes toxic, iodine excretion becomes elevated. In severe hyperthyroidism, after many months, the continued drain on the iodine reserves so exhausts the iodine supply as to interfere seriously with the function of the thyroid in manufacturing hormone. Therefore, the neglected case of exophthalmic goiter tends to show a lower BMR as the disease progresses. Such a patient ultimately may attain a normal plasma "hormonal" iodine (356) and the signs of increased caloric turnover may decline to a point where the patient's hands and feet become cold and the skin generally cool. The drain upon the thyroid's stores of iodine in hyperthyroidism is illustrated in Table VIII.

Studies of iodine metabolism in the clinic are sometimes complicated by extraneous factors. For example, a severe wasting illness will increase the

iodine excretion as the tissues are destroyed. This may occur after simple operations, after thyroidectomy or thoracoplasty (88), and has no definite significance with respect to thyroid function. This loss may amount to as much as 203 µg. of iodine in the first day or two after an operation. Of course, during a difficult removal of the thyroid, accidental massage by the surgeon may release rather large amounts of iodine from the gland itself. In fact, the protein thyroglobulin actually has been detected (255) in the thyroid veins and even in the general systemic blood immediately after drastic surgical manipulation of the gland. Similarly, x-ray therapy directed to the gland may produce an increased excretion of iodine (117), as may

TABLE VIII  
RENAL CLEARANCE OF INORGANIC IODIDE\*

Group	Presumably normal renal function				Presumably impaired renal function			
	Cases	Method A <sup>a</sup> cc. per min.	Method B <sup>b</sup> cc. per min.	Mean of individual differences cc. per min.	Cases	Method A cc. per min.	Method B cc. per min.	Mean of individual differences, cc. per min.
Hyperthyroid	10	38.4 ± 2.7 <sup>c</sup>	37.9 ± 2.2	+0.5 ± 2.8	9	28.2 ± 2.0	25.5 ± 2.6	+0.7 ± 3.1
Euthyroid	8	36.6 ± 3.3	36.0 ± 3.7	+0.6 ± 1.2	1	25.8	25.6	+0.2
Hypothyroid	5	22.3 ± 2.0	21.2 ± 1.8	+1.1 ± 0.7	1	13.4	16.4	-3.0

<sup>a</sup> Method A = Clearance estimated from standard equation  $\frac{UV}{S}$

<sup>b</sup> Method B = Clearance estimated from  $\frac{Q_u f \times r}{C_0}$ , as indicated in Fig. 21

<sup>c</sup> The values are the means and standard errors of the means.

\* Keating and Albert, *Recent Progress in Hormone Res.* 4, 453, (1949).

also the injection of radioiodide which accumulates in the gland and subsequently destroys it.

In summary, then, the thyroid regulates the concentration of circulating hormonal iodine in the body and at the same time acts to conserve and store iodide. As might be suspected, however, the gland itself is influenced by the presence of iodide in the body. Therefore, under certain circumstances iodide may be employed to alter thyroid function and development. It is convenient to recognize four levels of iodide intake which may influence thyroid physiology (411):—(1) the normal or physiologic iodine intake (2) prophylactic iodide therapy, (3) therapeutic iodine doses, and (4) "fibrolytic" iodine doses. The first of these has been already discussed in detail and need not concern us further.

In the case of the second, the dosage is about 1.1 mg. of total iodine per day (313). This is the amount which should be given a young person in an endemic goiter region to help her through normal adolescence. As this therapy is continued, the colloid increases somewhat and the follicular cells become flattened and less active functionally. The medication acts by diminishing the pituitary thyrotropic effect slightly, so that the tendency to hyperplasia which would result in goiter is diminished. Whether such dosages have a direct effect on the structure of the thyroid, or whether they act by suppressing pituitary secretion or by blocking the effect of pituitary secretion at the thyroid is unknown. Some physicians prefer to use thyroxine alone in this early stage, particularly if preexisting hyperplasia is present, because they fear that iodide given in excess may produce a sort of "Jod-Basedow effect" (371). Means (316) doubts the existence of such a phenomenon clinically, but Marine (518) has demonstrated it in rabbits. This problem will be discussed further in relation to Rawson's (374) theory of the dual action of iodine on the thyroid gland.

The total iodine content of the goitrous gland may vary considerably. For example, a gland which weighs three times the normal, e.g. over 70 g., may have a normal iodine content because of its increased mass of tissue even though the percentage of iodine in it is low. Characteristic figures would be 64 mg.% of iodine in the dry tissue for a normal gland as against only 44 mg.% in simple goiter. However, there is a very wide range of variation, and the iodine content may be influenced greatly by therapy.

One of the most striking effects of iodide on the thyroid is the characteristic suppression of function (365). The effect on the normal gland is so slight as to be debatable. In the hyperactive gland, however, the effect is so striking as to constitute a diagnostic test (320,29). In the treatment of exophthalmic goiter, large amounts of iodide are administered. It is not unusual to give from 10 to 75 mg. or more daily. Obviously these doses are huge compared with the physiologic requirement of less than 0.2 mg. daily. In the hyperplastic thyroid gland of Graves' disease, such doses have a remarkable effect both on thyroid anatomy and on thyroid function. Some of the many theories of this action of iodine have been reviewed recently by Rawson (374) who adds another of his own. Means (316) stated that thyrotoxicosis is subject to spontaneous fluctuations in degree, but that at any given activity the patient is somewhat better off if the circulating iodide is high. In other words, iodide tends to repress the elaboration of hormone or its release by the gland, no matter how severe the state of thyrotoxicosis. It is now clear that the effect of high concentrations of iodide is to block, in some degree at least, the secretion of the usual hormone (164,271), but some investigators believe that the inhibitory action involves not the thyroid, but the pituitary (140). Under successful iodide therapy the gland

is made to secrete internally into its follicles rather than externally into the blood stream (423). In other words an excess of iodide reverses the direction of flow of the hormone within the gland. With the advent of thiouracil, Rawson (374) has concluded that iodine exerts two actions upon the thyroid gland in Graves' disease. These are designated as a) iodinating action, and b) an involuting action. Through the use of thiouracil, these two actions can be separated. It must be confessed that we do not clearly understand why large doses of iodide produce a striking, if temporary amelioration of thyrotoxicosis of endogenous origin. It is clear, however, (454) that heavy dosage of iodide will not neutralize the thyrotoxicosis of exogenous origin.

The net result of high iodine dosage is to reverse the direction of hormone secretion for varying periods of time, averaging one to several weeks. During this period, a large amount of fresh thyroid hormone is manufactured, but is not secreted into the blood stream as usual. On the contrary, this new product is stored in the follicles. The author has termed this phenomenon endocretion to distinguish it from excretion. Eventually the follicles become distended with colloid and the gland becomes hard and tense to palpation and may even interfere with swallowing or breathing. Moreover, the vascularity of the gland is decreased. In the meantime the patient's supply of circulating thyroid hormone declines as rapidly as if the gland had been surgically removed (395). Thereupon the metabolic rate also declines in a semilogarithmic fashion much as if the patient were developing hypothyroidism at a hypernormal level (318). Eventually, when the follicles are overdistended with hormone, the secretion begins to overflow into the bloodstream. Obviously the physician may do a patient considerable harm by filling the gland up with fresh iodide stores and then sending him to a hospital for immediate operation. The mortality of thyroidectomy has been reduced in this century from as high as 50% to as low as 0.08% in some series (30,45,86), simply because the thyroid is removed while the patient is essentially in a euthyroid condition. At this stage, from a purely endocrinological standpoint, all the surgeon does is to lift an isolated thyroid out of the neck of an essentially normal patient.

In the milder grades of hyperthyroidism, it has been possible to maintain the patients in essentially a healthy condition for a number of years through the use of iodide alone (381). It would appear (320) that the presence of an excess of iodide tends to exert a constant backward pressure upon the secretion of thyroid hormone even at the higher levels of activity.

The anatomical and chemical changes which occur in the gland under such therapy are classical. In case of active exophthalmic goiter, the concentration of iodine per mg. of fresh thyroid tissue may be only 1.2 mg. per g. as compared with a minimum concentration in normal glands of 3.7. At this stage, practically no "I" fraction is present. Moreover, the "T"

fraction may be only 5-10% of the total. After the introduction of routine iodide therapy, the total iodine content of the gland may increase 25-fold within a week. During this time, the relative size of the "T" fraction rises steadily to an equilibrium proportion of more than 25% of the total. At first there is a relatively high percentage of inorganic iodine, but within a few days this fraction steadies down to about 10% of the total. While the plasma contains from 60 to several hundred  $\mu\text{g.}\%$  of iodide, protein-bound iodine is falling very rapidly during this time (395). This fall seems to occur a day or so ahead of the fall in BMR. In toxic nodular goiter the effect is very similar except that frequently the gland already is markedly enlarged. Because of this hyperplasia, the final total iodine content of the gland may amount to nearly 20 mg. or over twice the content of the normal thyroid.

In the "fibrolytic" iodine dosages such as once were used in the treatment of actinomycosis or of luetic gummata, one finds a somewhat depressing effect upon the thyroid. The follicles become well stored with colloid and,

of such tracer material equivalent to  $5 \times 10^{-11}$  g. of radium, suitably controlled Geiger-Müller counters would measure the radio-iodine with a standard error of 5%. This method is so sensitive that if a human individual is allowed to swallow a glass of radioactive iodide solution, and a Geiger counter laid against her thyroid, within five minutes the monitor connected with a radio amplifier will begin to emit irregular static noises. These increase in intensity for about fifty minutes, thus demonstrating the trapping of iodine within the gland. By suitable corrections and calibrations, the amount of radioactivity so measured can be correlated with the iodine actually found when the gland is later removed. Such observations were made by Hertz, Roberts and Salter (194) and by Hamilton and Soley (170).

One of the important features of work with radioiodine is the necessity for preliminary preparation of the material through the use of microbiological methods. For example, the radioactivity might reside either in the form of iodide or of diiodotyrosine or of thyroxine. Unless these have first been separated one from another it is of little interest that the thyroid contains iodine. A large number of tissues and body fluids can be studied, as shown in Fig. 23. This reservoir of iodine in the peripheral tissues is slowly converted into iodide and lost through the kidney eventually. Accordingly, whenever iodide has been administered, it has been found that the general tissue mass competed with the kidney clearance. For example, in such a study the excretion of iodide in the urine was found to be a semilogarithmic function of time (170). With the development of microchemical methods for iodine, however, it has now become possible to have simultaneous chemical and tracer iodine data. Salter and McKay (426) for example, have developed a catalytic determination of iodine applicable to amounts as low as 1/20th of one microgram.

It is impossible to pursue all the applications of this important method. It will be of interest, however, to cite a few observations in which the radioactive element gave interesting information. Hamilton and Soley (170) gave doses of 14 mg. of "labeled" iodide to human subjects by mouth before breakfast. They found that over 80% of the iodide so administered was absorbed by the digestive tract in one hour. As usual, a standard sample of the material was kept for calibration, and thereafter due allowance was made for the regular decay of the preparation. The urine was collected in 24 hour samples and the feces collected over a five day period. When a thyroidectomy was performed, e.g., three days after the administration of the radioiodine, the amount of thyroid tissue left by the surgeon was estimated and added to the total weight of tissue removed. In this way it was possible to collect simultaneous data on the thyroid and on the urine and feces and thus to derive a total metabolic balance.

Similarly Keating and Albert (227) studied the excretion of iodide through the kidney, using the classical formula for renal clearance,  $\frac{UV}{S}$ , which can

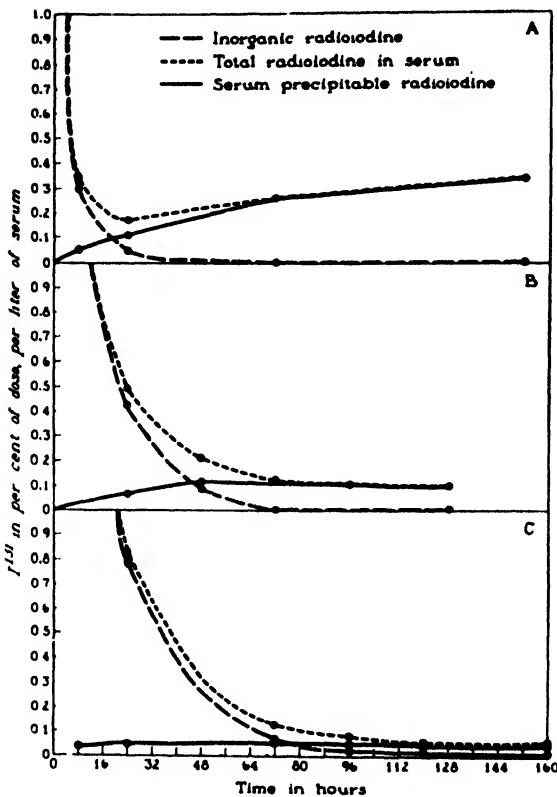


FIG. 23.—Inorganic and serum precipitable radioiodine in blood. When the radioiodine in blood is fractionated it is seen that the second phase represents the exponential disappearance of inorganic radioiodide and the third phase represents the appearance of significant quantities of organically bound radioiodine. A is a patient who has hyperthyroidism; B, a euthyroid patient with thyroid tumor; and C, a patient who has myxedema. (From Keating and Albert, *Recent Progress in Hormone Res.* 4, 429, 1949.)

be approximated by the following fraction (involving tagged iodine):

$$\frac{\text{Urinary excretion} \times \text{rate constant}}{\text{Blood concentration.}}$$

The values obtained by both methods compare satisfactorily both between themselves and with other values found in the literature. For example, a dog's kidneys cleared 9.0 ml. of plasma per minute (120). Likewise 13

normal men ingesting 88 mg. of iodide daily showed (227) renal iodide clearances of 10 to 25 ml. per minute. All of these data indicate that a definite temporary reabsorption of glomerular filtrate by the tubules can occur—with respect to iodide.

According to Keating and Albert (227), there is striking correspondence among the respective concentrations of tagged iodide in thyroid, blood and urine. The more tracer is trapped by the gland, the less spills out in the urine. The respective excretion rates, however, are much the same: in mathematical terms, the second derivative of either curve is nearly identical. If the fall in blood concentration of any given metabolite is exponential, the utilization of this substance is continuously proportional to the concentration in body fluids (109,101). Urinary excretion and thyroid storage of tagged iodide are both determined by the function  $[1 - e^{-rt}]$  and blood concentration by the function  $[e^{-rt}]$ ; i.e., the rate constant  $r$  is common to all three. Because the value for  $r$  applies to all the body fluids, it may be used to estimate approximately the following phenomena: 1) the volume of distribution of iodide; 2) renal function in terms of iodide excretion, 3) iodine trapping by the thyroid and 4) the utilization of iodide by extra-thyroidal tissues. By such calculations several interesting modalities are reached. For example, the distribution of radioiodide indicates a distribution volume of some 350 ml. per kg. in euthyroid man (227), corresponding to Wallace and Brodie's (516) value of 328 to 434 in the dog. In short, iodide is distributed largely in extracellular fluids.

Just before World War II broke upon us Corson, MacKenzie and Segre at the radiation laboratory in Berkeley, California prepared a new chemical element, No. 85. The existence of such a substance had been suspected from various compilations of the elements, but when it was finally obtained, the element seemed so much like a metal that some observers were dubious as to its proper classification. One of the most cogent reasons for calling it a halogen, finally, was that the element was preferentially trapped in the thyroid of the guinea pig! It is rather amusing to cite a biological behavior of this sort as evidence for regarding this substance as a member of the halogen family of elements; and for this reason Hastings suggested the name "thyrine" for this element. It has been rather puzzling that bromine and chlorine are not preferentially trapped in the gland. Therefore, it was the more surprising that the new material was so readily stored in the guinea pig's thyroid. The demonstration of this storage was undertaken by Hamilton (167) with a group of normal and of thyrotoxic guinea pigs. The thyroid hyperplasia was induced in the latter group by the daily administration of thyrotropic hormone for a week. At the end of this time the two groups received identical doses of radioiodine and of element 85. Groups of the thyrotoxic and of the normal animals were sacrificed at the end of 4 hours,

of 20 hours, and, later, of 65 hours. Urine and feces were collected during the period of study; and at autopsy the thyroid, lymph nodes, muscle, liver and blood were all sampled. It was found that the uptake of radio-iodine and element 85 was very small in the blood, lymph nodes, muscle and liver. Indeed the concentration in these tissues was less than one-hundredth the amount present in the thyroid gland. The thyroid, however, had preferentially trapped the new element, although somewhat less efficiently than iodine. At 65 hours, for example, normal animals showed 18.8% accumulation of iodine as against 4.5% for the element 85. Thyrotoxic animals showed 31.8% as compared with 8.8% for element 85. These observations indicate that element 85 is bound firmly in the thyroidal stores of hormone. Its official name is now "astatine."

Because of the very great affinity of goiters for iodine, it was suggested (191) that it might be possible to collect in the gland iodine of such high radioactivity that the resulting tissue damage would check the activity or the growth of goiters. By using 26-minute iodine concentrated in the thyroid, one might obtain a local effect without having any serious cumulative action on the body tissues at large. If the human thyroid collected iodine as well as the normal rabbit the dose to be desired in human patients with 75 g. of thyroid tissue was calculated to be 750 millicuries (191). Soley and Hamilton (473) have actually used radioiodine of high specific activity and have been so successful in inhibiting the action of exophthalmic goiters as to produce myxedema. Indeed the chief problem in this type of therapy at the moment is to avoid such excessive dosage that the gland is permanently destroyed. Since this section was written a considerable clinical experience has accumulated, which may now be reviewed.

This type of therapy is still in its infancy. It has several merits. The effect is insidious; the patient is ambulatory or, at least, no prolonged hospitalization is necessary. Even if a serious overdosage is given, the situation is no worse than the myxedema which is occasionally produced by the overenthusiastic surgeon. Moreover, the treatment can be repeated at intervals as needed. By means of  $I^{131}$  it is possible for about an hour's time to have within the thyroid itself the equivalent of a gram of radium. Moreover, this material is distributed diffusely throughout the organ and emits radiation of very high intensity. In the meantime, to be sure, some of the material is circulating in the rest of the organism; but in such low concentration and for such a brief period of time that it does no great damage. In addition, radioiodine also can be used in the clinic to study the metabolism of the thyroid itself in human subjects. A comparison of such data obtained with radioactive iodine has been compared with actual chemical analyses of the gland (194). In the myxedematous patient, obviously, no iodine is trapped. In certain isolated nodules of the thyroid, e.g., the true

adenomata, the ability of the tissue to pick up radioiodine may prove of diagnostic significance (77,370). Moreover, the intimate metabolism of iodine compounds within the gland has been studied with this technique (380,246,513).

Hertz and Roberts (189) reported a series of 29 cases of hyperthyroidism in which the chief treatment was internal radiation of the gland by radioactive iodine. In patients exhibiting goiters weighing approximately 60 to 75 g., 5 to 25 millicuries of radioiodide was highly effective in controlling the disease in about 80% of the cases. In general, the cases responded better if the glands were not already filled with inert iodine. Hamilton and Lawrence (169) and Chapman and Evans (66) made similar studies. In Chapman's series, between 14 and 79 millicuries of 12-day iodine,  $I^{130}$ , and about one-tenth of that amount of 8-day iodine,  $I^{131}$ , was given in 0.5 mg. of iodine as sodium iodide. The beta-rays emitted from such material, after its trapping in the thyroid, have a maximum scatter range of only a few millimeters of tissue. Consequently, the radiation is confined almost exclusively to the tissue containing the radioactive isotope. By giving a total dose per patient of about 50 millicuries, mainly in the form of 12-day iodine, it was possible to produce a tissue radiation of over 3000 roentgens, carried in about 1 mg. of ordinary iodide. About one-quarter of the patients had reactions resembling roentgen ray sickness. About one-sixth of the patients proceeded into myxedema after therapy.

In a symposium on radioiodine (339) in July, 1948, many of the important features of this subject were crystallized. Definite benefit has been derived by patients suffering from Graves' Disease and (to a lesser extent) from thyroid cancer. Chapman (68) recommended the eight-day isotope as most convenient as an effective single therapeutic agent in a dosage of 0.2 millicuries per estimated g. of thyroid. Because the average retention in toxic goiters is 71% of the dose, the active medication amounts to 0.14 millicuries per g. of tissue, and 97% of this energy is released within 30 days. Most patients respond satisfactorily to single doses between 4 and 14 millicuries, administered orally. A few patients exhibit an exacerbation of the thyrotoxicosis as the gland is injured by the radiation. Indeed, three weeks after such therapy the gland is edematous and the follicular cells (in histological sections) are separated from the basement membrane. A year later such glands show extensive fibrosis and regenerative hyperplasia. As yet no evidence is available that such lesions progress to thyroid cancer. Under radio-iodide treatment exophthalmos may or may not improve. No increase in exophthalmos has been precipitated. Among 155 patients so treated, 5 became accidentally myxedematous. Therefore it is clear that the beta-ray treatment is more effective than external X-ray therapy. As regards the latter, Menville (321) surveyed 10,541 cases and found that

66% were cured, 21% improved and 13% failed to respond. The danger of unsightly telangiectatic skin lesions as a sequel has limited the use of external gamma-ray treatment. Because the energy released from I<sup>131</sup> is nine-tenths in beta form, which penetrates only about 1 mm. through tissue, this complication is avoided. In another series of 46 patients, Soley (474) found the average uptake to be 95 microcuries per gram of gland among 27 successful responses; but the biological variation was very large. Of the 46 patients studied, 42 responded satisfactorily to a total dosage of 3.0 millicuries of I<sup>131</sup> and returned to normal function in about 3.4 months.

In respect to exophthalmos, two-thirds of the patients showed no change, and 9% had a marked increase. This increase in exophthalmos, therefore, is less than after surgical treatment but more severe than after external X-ray therapy. There was 3% as much iodine in the pituitary as in the thyroid some 19 days after therapy (68,192,521). Hertz (192) states that possibly once in 100 cases the hypothyroidism is permanent. Test doses of about 100 microcuries of I<sup>131</sup> in adult patients may be used to locate metastatic tissue (455) and to distinguish between functioning and nonfunctional tissue (421,422). In general (375), the morphologically anaplastic areas tend to eschew iodine. Most confusing, however, is the heterogeneity of single tumor masses. About 15% of cancer patients trap significant amounts of iodine in their thyroid-tumor masses (505). Among these, often about four-fifths of each tumor mass recedes, but despite continued trapping of the isotopes there is a tendency for the masses to recur and grow. Because of the low percentage of tumor masses which take up radioiodide, attempts have been made to improve the functional receptivity of metastatic tumors. Such attempts include total thyroidectomy, the use of thiouracil, the administration of thyrotropic pituitary hormone, and temporary alterations in renal activity. Thus far, no case of cure has been reported, but temporary and even striking relief has been noted in obviously hopeless cases, with very little attendant risk or discomfort. Seidlin (455) has reported the case of a man at 56 years of age, alive and without symptoms (referable to cancer) 5 years after the initial treatment with radioiodide. In patients without normal functioning tissue, 57% of the patients fixed radioiodide satisfactorily. This improvement possibly is due to the increased thyrotropic hormonal activity secondary to thyroidectomy or to destruction of the normal thyroid with beta radiation. The increased T.S.H. causes renewed growth and functional activity of latent metastatic tissue. Indeed, Seidlin (457) has reported two cases of hyperthyroidism arising from metastases and checked with thiouracil. If these active metastases are exposed to radioiodide, however, their very functional activity becomes a means of destroying them. Thus, a little radioiodide therapy may make the patient worse by destroying his normal gland and thereby arousing pituitary secretion. Persistent therapy with the isotope is indicated!

A unique feature of the use of tagged atoms is that the tissues may be examined in detail for the localization of the element (244,168). Various techniques are possible, (250,125) but a typical one is the following. The tissue is appropriately fixed in 10% formaldehyde and then paraffin sections are made varying from 3 to 5  $\mu$  in thickness. These are finally coated thinly with collodion and pressed against an Agfa No-screen x-ray film. They are held in place close to the film for a week or more as a rule. In consequence, the histological sections take their own pictures. In the meantime, from adjacent slices of the same tissue routine stained histological sections are prepared. In this way one obtains from the same tissue two simultaneous pictures:—a visual picture of the distribution of cells and various structures, and the invisible distribution of radioactive material within the gland (cf. Fig. 24). For example, by this technique it was shown that iodine accumulates rapidly within the follicle in the colloid and in a concentration greater than in the follicular cells themselves (171). In hyperplastic glands, indeed, iodine moves more rapidly into the colloid and in greater concentration than in the normal follicle. Thyroid neoplasms, e.g., thyroid carcinoma, often fail to fix iodine. In struma lymphomatosa, likewise, the large abnormal cells forming acini fail to fix iodine readily. Very large cysts may also be nonfunctional as indicated by the failure of the radioiodine to penetrate to the center. Almost no other technique exists which so clearly demonstrates the topographical distribution of a chemical. This correlating of morphology with function is one of the great contributions of the tracer technique.

In recent years attempts have been made to study the intimate metabolism of the thyroid gland. When the thyroid happened to be emptied of iodine, as in the very hyperplastic gland of untreated human Graves' disease or in similar glands produced by massive injections of thyrotropic hormone into guinea pigs, radioiodine rapidly accumulated in the gland. When such a gland was fractioned by the usual chemical procedure, the iodine could be divided into the three characteristic fractions namely "I" (inorganic) iodine, "D" (diiodotyrosine-like) iodine, and "T" (thyroxine-like) iodine. When simultaneous measurements of these fractions were made by the Geiger-Müller counter it was possible to demonstrate (194) that the values corresponded rather well, as would be expected from the more laborious but better tried biochemical technique. Monoiodotyrosine, if present, falls into the diiodotyrosine fraction.

More recently this procedure has been applied to study the transformation of iodine into diiodotyrosine and subsequently into thyroxine (328,357, 250). Working with very small amounts of tissue, such as surviving slices of animal thyroid, as shown in Table IX, it has been possible to follow iodine uptake (Table IX) and also ratios of among the three fractions. For example, after three hours the distribution I:D:T fractions was as 1:6:1.

Chaikoff isolated each of the "D" and "T" forms as a pure substance from a mixture of its radioactive and nonradioactive forms and recrystallized

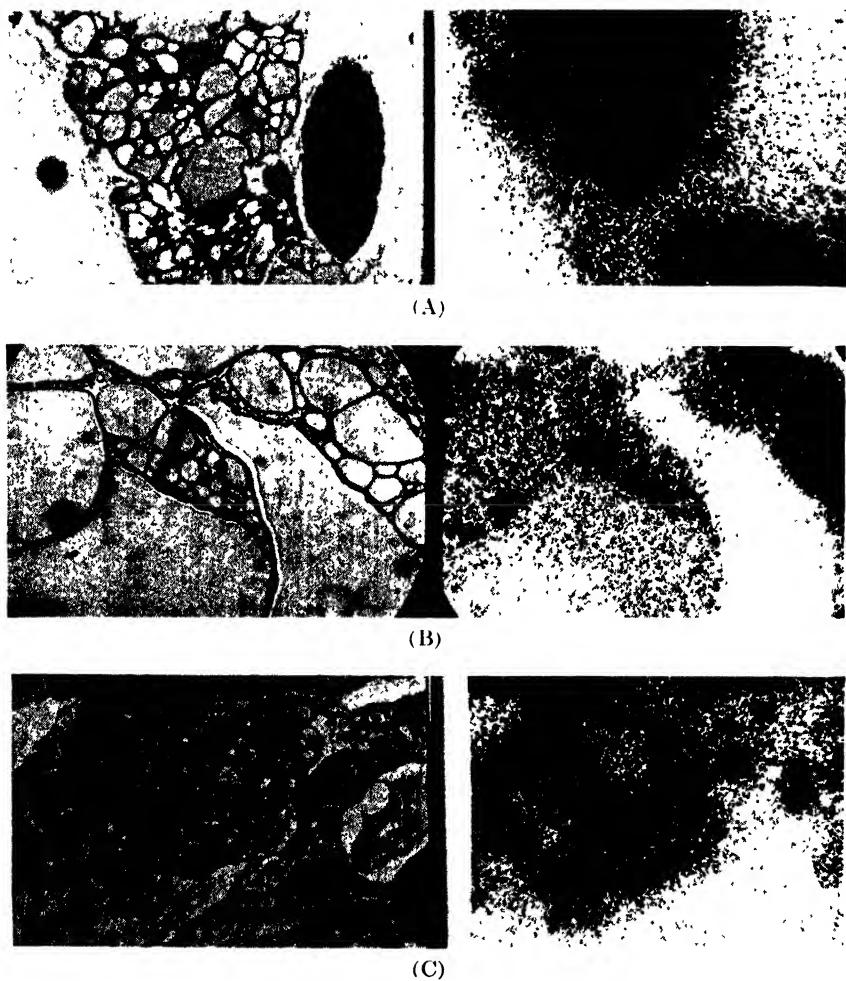


FIG. 24.—Comparison of radioautographs (right) with routine histological sections (left) in three types of thyroid tissue. (A) Normal thyroid with parathyroid adjacent. (B) Nontoxic goiter. (C) Primary hyperplasia. (By courtesy of Dr. J. G. Hamilton.)

these to constant specific activity. When the tissue was homogenized, it failed to transform  $I^{131}$  iodide into "D" and "T" forms.

A difficulty in the use of radioiodine which must be taken into account is the problem of atomic interchange. It is very important when using tracer atoms to complete the experiments at least once with microanalytical

TABLE IX  
IODINE CONCENTRATION BY SURVIVING LIVER, SPLEEN, AND THYROID SLICES<sup>a</sup>

Tissue	Iodide $I_{131}$ added to Ringer's, $\mu g.$	Time of incubation, hours	Per cent of Ringer's $I^*$ recovered in slices	Micrograms of Ringer's $I_{131}$ incorporated into slices
Sheep liver	0 <sup>b</sup>	1	2.4	
Sheep thyroid	0 <sup>b</sup>	1	81.4	<0.3
Beef spleen	0 <sup>b</sup>	2	1.5	
Beef thyroid	0 <sup>b</sup>	2	87.9	<0.3
Sheep thyroid	0 <sup>b</sup>	1	86.2	<1.3
Sheep thyroid	20	1	61.3	12

<sup>a</sup> From Chaikoff and Taurog. Studies on Iodine Compounds, in Thyroid Function as Disclosed by Newer Methods of Study. J. H. Means, editor. Ann. N. Y. Acad. Sci. 50, 381 (1949).

<sup>b</sup> When no iodide was added, the Ringer's medium contained less than 0.3  $\mu g.$  of iodine.

\* Radioactive.

TABLE X  
ATOMIC EXCHANGE BETWEEN INERT DIODOTYROSINE AND RADIO-ACTIVE  
POTASSIUM IODIDE  
(A Confusing Experimental Artifact)

Temp., 25°C.	Radioactivity in organic iodine	Radioactivity in inorganic iodine	Total radioactivity
	Start with equal parts of		
Stable isotope as diiodotyrosine	Radioactive isotope as iodide		
	0%	100%	100%
Acid, 40 min.....	30%	67%	97%
Acid, 18 hrs.....	32%	70%	102%
Alkaline, 40 min.....	4%	96%	99%
Alkaline, 18 hrs.....	2%	96%	98%

Excerpt from studies in progress with the collaboration of Dr. Wendall C. Peacock of the Massachusetts Institute of Technology.

procedures of a purely chemical nature. Thus one can decide whether there exists a danger of confusing physiological exchange with atomic interchange, in studies involving cell metabolism (see 325,415). Recent work (416) sug-

gests that under controlled conditions, such as the avoidance of marked acidity, the difficulty may possibly be obviated in the laboratory manipulation of iodine. The author has demonstrated that in acid solution a very rapid interchange may occur so that one third of the iodide may apparently be transformed into organic iodine compounds, whereas actually no change in the chemical equilibrium has occurred (See Table X). This problem has been considered in relation to biological studies by Chaikoff and Taurog (65) and by Astwood (19), who have concluded that under suitable precautions the error need not be over 5%.

Within the cell, however, under the influence of enzymes, it is conceivable that a dynamic equilibrium between various intracellular substances is established. In this way, although the net metabolism of the cell might be essentially static, nevertheless there might be a very active interchange of atoms or chemical groups. A further danger is the possible damage to intracellular enzyme system from highly radioactive materials such as have become available recently.

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CHAPTER V  
The Control of Thyroid Activity  
By WILLIAM T. SALTER

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**I. Physiology of Thyrotrophin**

**A. THE PITUITARY THYROTROPHIN**

In the preceding chapter the pituitary thyrotrophin has been discussed in connection with its role as the chemical intermediary of the pituitary-thyroid axis. It has been made clear that the thyroid, the "target" gland for which the thyrotrophin is extraordinarily specific, depends upon tonic and continuous stimulation by this substance for the maintenance of its activity. Many of the most important reactions of the thyroid gland to the varied circumstances and strains of life depend upon the intervention of the thyrotrophin. These circumstances have been discussed somewhat in detail in the preceding article. It is the purpose of this later section, herefore, to dwell in greater detail upon the properties of the thyrotrophin

itself; to discuss possible mechanisms by which it interdigitates with the parenchyma of the thyroid; and in particular to consider its relationship with the thyroid blocking agents which have become so important in clinical therapy as well as in laboratory experimentation. It will be seen that the effect of goitrogens is dependent in large measure upon the activity of the thyrotrophin, and that, indeed, there seems to be a constant balance between goitrogenic substances and thyrotrophin within the organism. Even in the absence of the pituitary, however, the thyroid shows a small but continuous activity in its own right.

#### B. QUALITATIVE IDENTIFICATION OF THE THYROTROPHIN

As discussed by Adams (2) the thyrotrophin is found in many classes of vertebrates and its locus is limited to the anterior lobe of the pituitary. Whether it is formed by acidophilic or basophilic cells, however, is still undecided (138). No other tissue yields extracts which have its highly specific property of stimulating the thyroid directly, as pointed out by Sturm and Schöning (145). The hormone is frequently considered as a biological entity, but preparations may differ both in their chemical and in their physiological behavior. For example, extracts have been described which show a preferential effect either in causing cellular hypertrophy or hyperplasia and on the other hand of increasing the weight of the thyroid (64). Similarly Dobyns (41) has described the fractionation of anterior pituitary extracts to yield two preparations: the first, highly effective in producing glandular hypertrophy, the second notable for its effect in producing exophthalmos. Likewise, other fractions have been described such as a heat-stable preparation which expedites the metamorphosis of tadpoles and a heat-labile fraction which stimulates the thyroid of mammals. In addition to these biological distinctions there is, of course, the ever-present species difference. Because the thyrotrophin is protein in nature so far as we now know, animals or men injected with moderate doses thereof will ultimately develop an immunity to the preparation so that it becomes inert (57). Moreover, as pointed out by Dvoskin (43), evidence of thyrotrophin activity may be simulated falsely by substances which put a strain upon the thyro-pituitary axis.

The thyrotrophin is a glycoprotein containing nearly 13% nitrogen, 6% hydrogen, 42% carbon, and 2.5% glucosamine. In many respects it behaves like a globulin with a rather broad isoelectric zone. The preparations obtained by Abraham White and Ciereszko showed a molecular weight around 10,000 with an activity of one chick unit per microgram. Its biological activity is destroyed by heating and by proteolysis with pepsin, trypsin, and chymotrypsin. It is also inactivated by oxidizing agents, like potassium permanganate and elementary iodine.

### C. ACTION OF THYROTROPHIN *in vitro*

In order to eliminate the possible effects of nervous stimulation and of changes in circulation, various investigators have studied the influence of preparations of thyrotrophin upon thyroid cells or surviving fragments of the thyroid gland *in vitro*. Classical studies of this type were made by Eitel, Krebs and Loeser (45) as early as 1933. Later Foote, Baker and Carrel (47a) used the Lindberg heart to perfuse whole human glands. When thyrotrophin was added to such preparations, characteristic evidence of activation occurred. Recently Junqueira (66a) has maintained fragments of the thyroid glands of white rats in Carrel flasks under oxygen as described by Parker (108). These fragments appeared to be reasonably healthy for as long as 96 hours. They were studied by the freezing-drying technique of Altman-Gersh (56) as modified by de Robertis (39). Within 30 minutes after thyrotrophin was introduced into the system the amount of intracellular colloid increased markedly. Later the secretion of colloid droplets into the follicular lumen was accentuated and the release of intrafollicular stored colloid became evident. The ingestion of released colloid by macrophages was suggested. When sodium iodide was added in concentrations from 0.15 M to 0.015 M, definite inhibition of secretion and of the release of colloid resulted. Two possible explanations of this peripheral action of iodide upon the thyroid gland have been suggested. The first is an inhibition of intracellular enzyme systems. The second is the inactivation of thyrotrophin. With respect to the proteolytic enzyme system within the follicular lumen, De Robertis and Nowinski (35,40), have suggested a direct inhibition of this enzyme by iodide. A similar hypothesis has been advanced by Dziemian (44). The problem is complicated, however, by several other interrelated observations. For example, the studies of Chaikoff and his colleagues, to be cited later, show that in the presence of sulfa drugs iodide can not be converted into thyroxine, even though thyrotrophin acts powerfully upon the poisoned thyroid. Moreover, the observations *in vivo* of Friedgood (53) and of Loeser and Thompson (80a) have shown that iodide interferes with the action of thyrotrophin. In addition, Rawson, Sterne and Aub (113a) found that explants of thyroid tissue *per se* can inactivate thyrotrophin. Rawson and his colleagues (110) have finally extended these observations to include clinical effects of iodide therapy. Thus, even on the thyroid gland, not to mention the pituitary gland, iodide may conceivably have a dual influence. This group of investigators (5) showed further that the loss of thyrotrophic activity produced in a pituitary extract by exposure to elementary iodine, could be restored (more or less) by treating the iodinated material with reducing compounds, among which were several goitrogens. These included 2-thio-

uracil, 6-N-propylthiouracil, aminothiazole, 5-amino-2 mercaptothiadiazole, 3-phenylaminomethyl-2-mercaptopthiazoline and potassium thiocyanate. In addition, the exposure of a pituitary extract to these compounds led to an augmentation of its thyrotrophic activity. The augmented potency of the thyrotrophic preparation persisted even after removal of the excess of goitrogen.

Albert and Rawson (4) have studied the effect of elementary iodine upon active preparations. For example, they mixed 20 mg. of crude thyrotrophic extract with 10 ml. of compound solution of iodine, and incubated the mixture for 100 minutes at 25°C. Most of the protein-bound iodine appeared in the precipitate which at pH 5.0 contained 10% of iodine; and the activity of the preparation disappeared. If less free iodine were used, less inactivation occurred. When this iodinated material was treated with certain reagents, a considerable proportion of the original activity was restored. These materials were in the main reducing agents. As this reactivation occurred the insoluble (but suspended) material dissolved to form an aqueous solution. Moreover, it appeared that certain of the goitrogenic agents, when added to a solution of thyrotrophin, increased its biological effect. This augmentation could occur even though the excess of the goitrogen were dialyzed away before administering the thyrotrophin to the test animal. These experiments are, of course, preliminary because pure thyrotrophic hormone is not yet available for study. They are of interest, however, in connection with the "dual theory of iodine action" of Rawson (111) which will be discussed later.

#### D. BIOLOGICAL CONCENTRATIONS OF THYROTROPHIN

Adams (2) has listed the contents of thyrotrophin in the anterior pituitary lobe of various species. Beginning with the least amount the sequence is as follows: the hen, guinea pig, chick, pigeon, cat, rabbit, horse, man, turkey, ox, toad, sheep, pig, dog, mouse, rat, sole, and frog. Indeed the histological appearance of the pituitary seems to be generally related to the thyrotrophic concentration. When the thyroid gland appears active, the concentration of thyrotrophin in the pituitary is generally great. This observation agrees with the findings of Salter, Cortell and McKay (132) who studied the pituitaries of rats under treatment with a goitrogen. The histological appearance of these glands, before and after the use of thiouracil can be summarized as follows:

In those animals receiving thiouracil alone, the drug produced many basophils and caused eosinophils nearly to disappear. The basophils increased both in number and in size. Most of them exhibited typical vacuolization and blue-staining hyaline material within the vacuoles. The chromophobes were somewhat increased in number. These changes are characteristic of those described by Sevringshaus, Smelser and Clark (139) Zeckwer *et al.* (167) and Guyer and Claus (61) following complete

thyroidectomy in the rat. Similar changes were found by Mackenzie and Mackenzie (84) and Griesbach and Purves (60) following treatment with a goitrogenic agent. The addition of potassium iodide had little effect on the picture, although a few faintly acidophilic granules appeared in some of the chromophobes.

When thiouracil was stopped and chow (alone) resumed, the basophils disappeared gradually and the eosinophils appeared rapidly. On the eighth day after stopping thiouracil there was a complete return of acidophilic granules but the basophils were still increased in size and number. Thereafter, up to the twenty-second day, the number and size of the basophils gradually declined, as did the extent of vacuolization. The addition of potassium iodide to the chow did not significantly alter the return to normal conditions.

When the thiouracil diet was exchanged for thiocyanate, the transformation toward the normal occurred only gradually. On the eighth day the acidophilic granules were still markedly depleted, but had returned to normal on the sixteenth day of thiocyanate treatment. Throughout this treatment, however, the basophils continued to be increased in size and number and to show a high degree of vacuolization. When the thiocyanate was supplemented with potassium iodide no significant effect on the previous picture was noted. On the twelfth day of this combined treatment the acidophils had become normal, but vacuolized basophils were prominent.

Such evidence suggests that the basophils manufacture thyrotrophin.

As pointed out by Albert (3) the most sensitive animals for the assay of exogenous thyrotrophin should be animals like the young chick and the guinea pig which have less active thyroids and low thyrotrophin concentrations in their anterior pituitaries. Similarly the ineffectiveness of goitrogens in the silver strain of dwarf mice might be explained on the basis of low thyrotrophin production by the anterior pituitary of these animals. Other variations in the activity of concentration of the thyrotrophin are also produced by such factors as reproductive activity, season, diet and cold environments.

#### E. BIOLOGICAL EFFECTS OF THYROTROPHIN

In a previous section many of the effects of the thyrotrophin have been described. These include hypertrophy and hyperplasia of the follicular cells, together with changes in the mitochondria and Golgi apparatus of the individual cell with a reversal of polarity as described by Bensley (23) and De Robertis (120). One of the features of this change is an increase in the water content of the thyroid parenchyma. Soon afterwards there occur characteristic changes in the colloid, particularly vascularization and reabsorption of the thyroglobulin; together with a bluish change in the previously eosinophilic protein as viewed by the usual staining methods. At the same time there occurs an increase in the protease activity in the contents of the follicle. The next effect is a loss of the stored organic iodine; but in spite of this loss there is an increase in the vascularity of the gland. Therefore, the size of the gland increases. Grossly it becomes hemorrhagic

and friable. As this activity goes on, the increased release of thyroid hormone produces the characteristic effects to be expected from the peripherally acting substance, thyroxine. This substance naturally causes a rise in the basal metabolic rate with a loss of liver glycogen in mammals or an

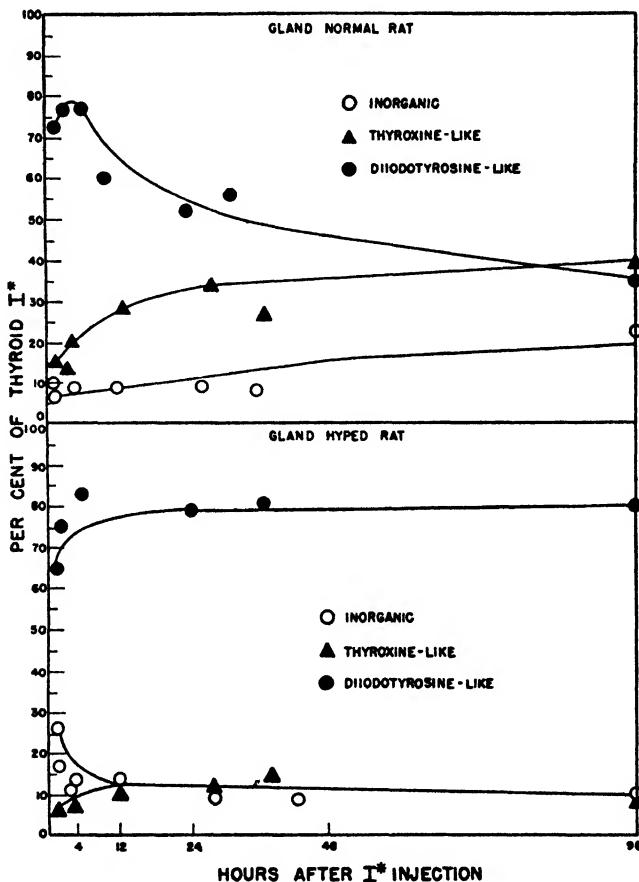


FIG. 1.—The distribution of thyroid I\* in normal and hypophysectomized rats. Each rat received intraperitoneally a tracer dose of I\*. (From Chaikoff and Taurog, Studies on Iodine Compounds, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 396, 1949.)

increased rate of metamorphosis of tadpoles and axolotl. That such phenomena are not due to the thyrotrophin *per se*, can be shown by the simultaneous administration of goitrogen. Once the stored colloid has been exhausted, further use of the goitrogen will not produce characteristic thyroxine effects because the synthetic processes which produce thyroxine within the goitrogen-poisoned gland fail to occur. Not all of the thyroid's

responses to the pituitary secretion occur simultaneously. Rawson (109) has observed that a loss of thyroid iodine precedes the increase in the mass of acinar cells; and that an increased capacity of the thyroid to trap iodine follows only at a later interval. As shown in Fig. 1, the thyrotrophin affects thyroidal iodine considerably. From the standpoint of iodine metabolism the changes produced by the thyrotrophin can be divided into

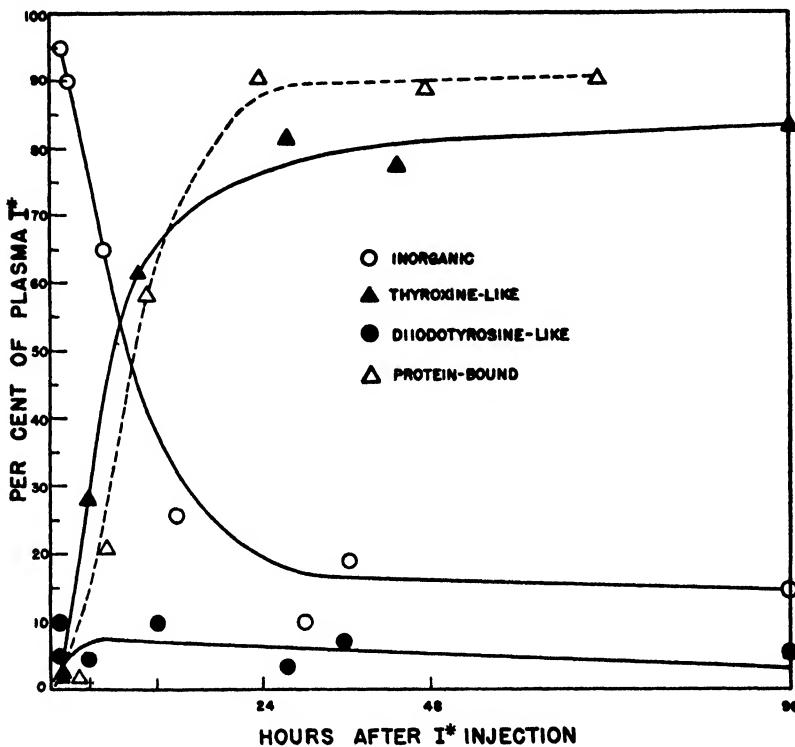


FIG. 2.—The distribution of plasma  $I^*$  among the various iodine fractions. (From Chaikoff and Taurog, Studies on Iodine Compounds, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 392, 1949.)

those involving the thyroid gland itself and those reflecting the subsequent distribution of the output of the gland. As shown in Fig. 1 the uptake of iodine by the thyroid gland is quite different in normal and hypophysectomized rats. This feature applies not only to total iodine in the gland, but also to the several chemical fractions within it and are illustrated in the figure. Subsequently, these effects are reflected in the iodine content of the plasma as shown in Figs. 2 and 3, taken from the work of Chaikoff

and his colleagues. Indeed, as shown in Table I, both rats and guinea pigs show a striking difference in the specific radioactivity of the protein-bound iodine within three hours after injection. This effect can also be noted in peripheral tissues like the intestine within a day after thyrotrophic administration to guinea pigs.

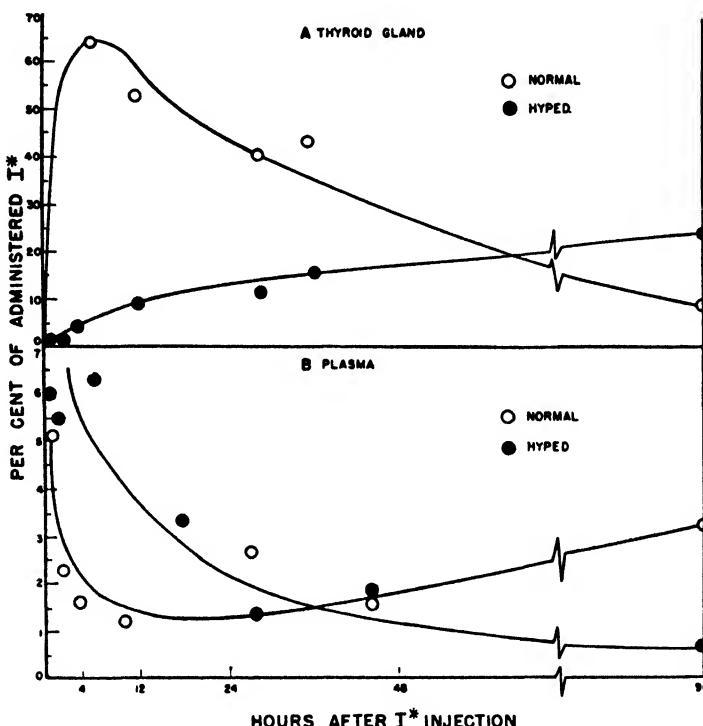


FIG. 3.—The uptake of total  $I^*$  by the whole thyroid gland (A) and by entire plasma (B) of the normal and hypophysectomized rat. Entire plasma was estimated as 5% of the total body weight. A tracer dose of  $I^*$  was injected intraperitoneally into each rat. (From Chaikoff and Taurog, Studies on Iodine Compounds, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 395, 1949.)

The collection and fixation of iodide by the thyroid is a function of the thyroid tissue itself and does not depend directly or exclusively upon thyrotrophic stimulation. For example, Morton *et al.* (106) studied the thyroids of hypophysectomized rats and found that they collected considerable amounts of iodine although somewhat less than did the normal controls. In studies on surviving slices of thyroid, also, the fixation of iodine was observed by Chaikoff and his collaborators. It is interesting, however, that the thyroids of their hypophysectomized rats formed very

little thyroxine from the diiodotyrosine present. These results are illustrated in Figs. 1 and 4. It would appear that the thyrotrophin has at least two chief effects upon the thyroid. These are (a) the liberation of

TABLE I  
FORMATION OF PLASMA PROTEIN-BOUND I\* BY RATS AND GUINEA PIGS INJECTED WITH THYROTROPIC HORMONE<sup>a</sup>

Animal	Interval after I* injection, hours	Controls			Thyrotropic hormone <sup>b</sup>		
		Weight of thyroid glands, mg.	Protein-bound I* as per cent of injected dose per 10 cc. plasma	Protein-bound I* × 100 / Total I*	Weight of thyroid glands, mg.	Protein-bound I* as per cent of injected dose per 10 cc. plasma	Protein-bound I* × 100 / Total I*
Rats	3	23	0.29	7.3	36	2.9	73
	3	22.5	0.21	5.2	30	1.3	39
	3	29	0.17	4.1	76	0.73	53
	3	33.5	0.26	9.2			
	6	31.5	0.44	30	39	2.0	82
	6	19	0.36	11	49	4.1	88
	6	33	0.34	27	43.5	3.1	87
	6	38	0.31	32			
Guinea pigs	16	75	0.0041	7.5	113	0.070	77
	16	56	0.0045	12	147	0.14	83
	16	68	0.0031	12	106	0.11	77
	16	68	0.0064	18	120	0.13	81
					112	0.099	86

<sup>a</sup> From Chaikoff, I. L. and Taurog, A. Studies on Iodine Compounds, in "Thyroid Function as Disclosed by Newer Methods of Study." *Ann. N. Y. Acad. Sci.* 50, 390 (1949).

<sup>b</sup> The rats were injected with 10 mg. of thyrotrophic hormone twice daily for 4 days. The 3-hour and 6-hour groups also received one injection on the 5th day at the same time that the I\* was injected. The guinea pigs received 2 mg. of thyrotrophic hormone injected once daily for 3 days and twice on the fourth day. They were sacrificed on the fifth day.

colloid already stored within the follicle, including the constituent hormone contained therein; and (b) an increase in the thyroxine-manufacturing apparatus present in the acinar cells which line the follicle. Whenever an increase in thyroid hypertrophy occurs, a parallel increase in the collection

of radioactive iodine will ensue, as shown by Hertz (63). Conversely, as shown by Leblond and Süe (76), the uptake of tracer iodine is much less in hypophysectomized animals; whereas it is greater than normal in animals

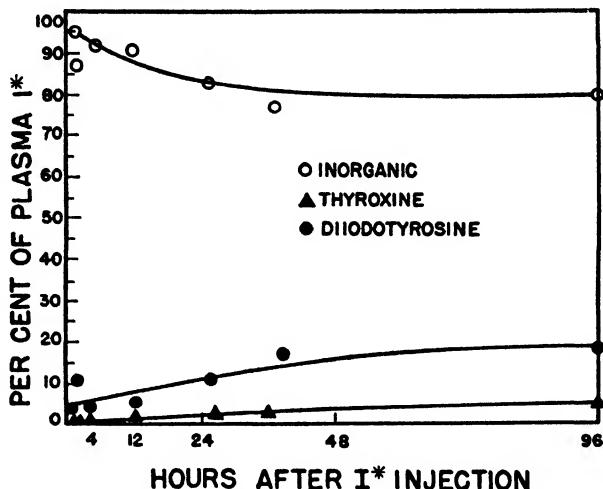


FIG. 4.—The distribution of plasma  $I^*$  in the hypophysectomized rat. (From Chaikoff and Taurog, Studies on Iodine Compounds, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* **50**, 397, 1949.)

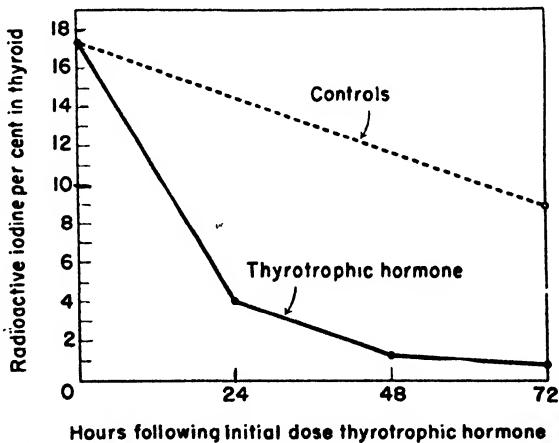


FIG. 5.—Hours following initial dose thyrotrophic hormone. (From Rawson, The Thyroid Stimulating Hormone, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* **50**, 493, 1949.)

treated with thyrotrophin. In studies with radioactive iodine in young chicks, Keating (68) found that within 24 hours after the administration of thyrotrophin over three-fourths of the radioactive iodine had disappeared, as shown in Fig. 5. By this time, the cell height of the average

acinar cell had nearly doubled; but a considerable increase in the weight of the gland was not noted until after 72 hours of treatment with daily injections of one J-S unit of thyrotrophin *per diem*. After the initial purging of previously stored iodine, there was a subsequent upswing of the iodine content of these thyroid glands. It was first evident after 48 hours of treatment. After four days of treatment the labeled iodine had increased fivefold as shown in Fig. 6. This early loss of thyroid iodine was found to occur in Rawson's observations (109) as early as 6 hours after the injection of the thyrotrophin, with a maximum loss at 24 hours. The greatest mean

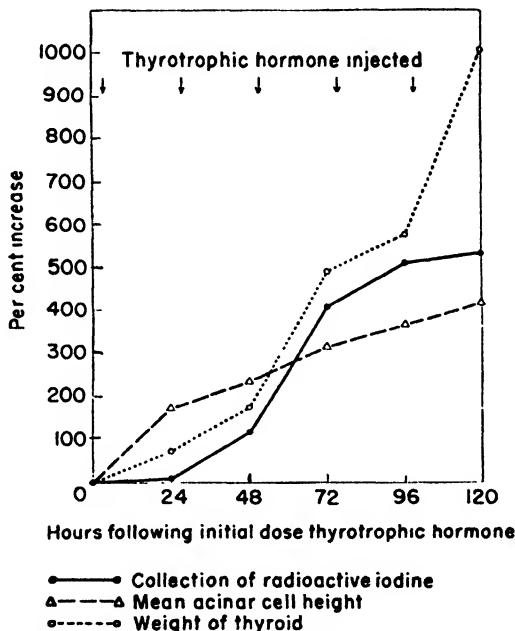


FIG. 6.—From Rawson, Physiological Reactions of the Thyroid-Stimulating Hormone, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* **40**, 492, 1949.

cell height was not reached until 30 hours, i.e., some 6 hours after the minimal iodine concentration had been noted. These observations are consistent with the finding of De Robertis (38) that the proteolytic activity within the thyroid follicles increases soon after the administration of the thyrotrophin. It is evident, therefore, that the thyrotrophin first liberates stored hormone from the follicular lumen and only later increases the manufacture of new hormone by enlarging the cellular enzymic machinery.

Rawson and his colleagues (109) have studied the relation of thyrotrophin to surviving thyroid tissue. In these studies not only slices of the gland

were used but also tissue cultures of explanted thyroid cells. The outstanding effect of this surviving thyroid tissue is its ability to inactivate thyrotrophin. For example, a normal rabbit thyroid will inactivate 12 units of thyrotrophin whereas other tissues have relatively little effect upon the activity of the medium. The exceptions are lymphatic and thymic tissue which can inactivate five units of the hormone on a similar basis, as shown in Fig. 7. This finding is interesting in view of the relationship of lymphatic tissue to Graves' disease described in an earlier section of the preceding article. Dobyns has studied the effect of preparations of thyrotrophin on thyroidectomized guinea pigs with respect to the production of exophthalmos. In similar experiments conducted in collaboration with

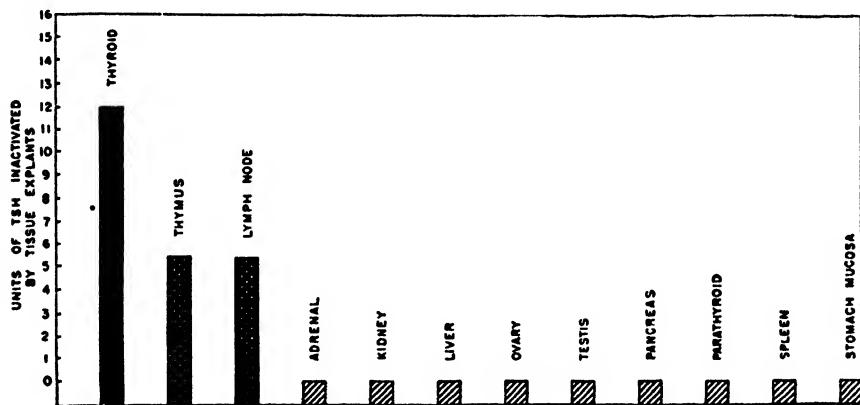


FIG. 7.—The amount of thyroid-stimulating hormone inactivated under optimal condition by the tissues indicated. (From Rawson, The Thyroid Stimulating Hormone, in *Thyroid Function as Disclosed by Newer Methods of Study*. *Ann. N. Y. Acad. Sci.* 50, 498, 1949.)

Rawson it was found that the exophthalmos-producing effect was annulled on incubation with thyroid tissue. Experiments were made also with excised human tissue. Explants of nodular thyroids failed to inactivate the thyrotrophin in the medium, whereas normal human tissue inactivated nearly 4 units of the hormone. Moreover similar explants from Graves' disease (primary hyperplasia) inactivated some seven units of the thyrotrophin. Control experiments with gonadotrophin failed to show any inactivation with thyroid tissue. The interpretation given by Rawson (115) to these observations is that the thyrotrophin participates in an oxidation-reduction mechanism as it exerts its action on the metabolism of thyroid tissue. In this connection it is interesting that when the thyrotrophin has been inactivated by exposure to thyroid tissue, it can be largely re-activated by treatment with reducing agents which are also goitrogenic.

Such experiments are positive, for example, with thiouracil, 5-aminothioldiazole and 3-phenylaminomethylthiazolidine-2-thione. Rawson also found that when iodide (100 mg.%) was added to the medium, relatively little thyrotrophin was inactivated by the normal rabbit thyroid. This observation suggests that an excess of iodide can interpose some sort of block between the thyrotrophin and the thyroid acinar cell.

It is now well established that both iodide and thyroxine will interfere with the effect of *exogenous* thyrotrophin on the thyroid of the hypophysectomized rat. This effect was demonstrated by Cameron and Carmichael (26) as early as 1920. It has been studied further by Loeb (80), by Loeser (81) and by Cortell and Rawson (32). In brief, the problem started with the observation that when iodide or thyroxine is administered to normal rats, the thyroid glands undergo involution to an extreme resting state. Formerly it was supposed that this effect was entirely due to a suppression of pituitary activity. The experiments of Cortell and Rawson, however, indicate that when 4 J-S units of thyrotrophin was injected daily for 4 days in rats receiving 20  $\mu$ g. of thyroxine for 10 days, the reaction of the thyroid cells in such animals was suppressed. Much the same result can be obtained by giving iodide. It must be assumed therefore, that the effects of iodide and thyroxine are not exclusively central upon the pituitary but that these substances also exert a peripheral effect between the gland and the thyrotrophin itself. Obviously, the rats just described had been hypophysectomized.

De Robertis (36) measured the proteolytic activity of normal and pathological glands photometrically by determining the rate at which tyrosine and tryptophan were split off from the protein edestin. In goiters from severely thyrotoxic patients the proteolytic activity was nearly twice (i.e., +96%) that of the average normal gland. Simple goiters had an elevation of +28%. In mild thyrotoxicosis which had responded to iodide therapy, the activity was -26% below the average normal level. In toxic adenomatous goiters, the proteolytic activity of the active nodules was twice that of the surrounding quiescent parenchyma.

In the light of the rather gross nature of present methods for detecting thyrotrophin, Albert (3) concludes that the thyrotrophin exists in body fluids only in traces. In fact it might be inferred that human serum contains about 0.5 J-S unit/100 ml. Likewise a rough estimate might be that normal human urine contains about 0.5 unit/100 ml. of urine. So contradictory, however, are various results in the literature, as shown in Table II that it cannot be asserted with confidence that even a qualitative identification of the thyrotrophin has been made in blood or urine.

When rats are subject to thyroidectomy, a definite fall in the concentration of "hormonal" iodine in the blood plasma occurs within 4 hours. In

three days time it declines from a normal level of about 3.5 to less than 1  $\mu\text{g}$ . per cent. In such animals, as reported by Chaikoff, Taurog and Reinhardt (28), the incorporation of injected radioiodine into the plasma is retarded. Indeed, Salter and Johnston (133) have pointed out that the blood plasma can be regarded as a true tissue in which thyroid hormone "turns over" as in other tissues with progressive metabolic changes. When thyrotrophin is injected into rats and guinea pigs, the opposite trends are found. The plasma "hormonal" iodine increases. Moreover, when radioiodide tracer is injected into animals receiving injections of thyrotrophin, the

TABLE II  
SUMMARY OF SOME TESTS FOR TSH IN BLOOD SERUM (WITH CHEMICAL TREATMENT  
OF SERUM)<sup>a</sup>

Reference	Source	Treatment	Volume per test animal, ml.	Method of assay	Re-sults
147-148	Rabbit, human (n.) <sup>b</sup>	Acetone ppt.	10	Guinea pig, weight	0
	Human (thy.)	Acetone ppt.	10	Guinea pig, weight	0
	Human (myx.)	Acetone ppt.	10	Guinea pig, weight	0
	Rabbit (thyroidec-tomy)	Acetone ppt.	10	Guinea pig, weight	+
149-151	Human (n., thy., myx.)	Acetone ppt.	10	Guinea pig, histo-logic	+

<sup>a</sup> From Albert, A. Biochemistry of Thyrotropic Hormone, in "Thyroid Function as Disclosed by Newer Methods of Study." *Ann. N. Y. Acad. Sci.* **50**, 477 (1949).

<sup>b</sup> Explanation of abbreviations: n. = normal; thy. = thyrotoxicosis; myx. = myxedema; ppt. = precipitate.

rate at which the tracer appears in the protein-bound fraction of the plasma is greatly increased. In some instances the specific radioactivity of the circulating hormone increases as much as tenfold. Salter (125) has pointed out that this coefficient is a useful measure of the rate of metabolic activity. The same procedure has been applied by Taurog, Chaikoff and Entenman (147) to dogs. In dogs weighing about 9 kilos, the turnover rate of the plasma "hormonal" iodine lay between 50 and 100  $\mu\text{g}./24$  hours. From these values it could be calculated that the virtual turnover time for the entire circulating plasma was between 4 and 7.5 hours. Somewhat similar studies were made by Schultze and Turner (136) in goats with an average

weight of 21.8 pounds. In these animals the rate of thyroxine secretion of the thyroid gland was computed to be equivalent to 59  $\mu\text{g}.$ /day of iodine as found in *l*-thyroxine. The rapid deposition of radioiodine in the thyroids of guinea pigs treated with thyrotrophin was observed by Leblond and Gross (75), who used the "coated autograph" technique. The tracer iodide was first fixed in the cells, the polarity of which was in the direction of secretion into the follicle.

That thyroxine can depress the effectiveness of exogenous thyrotrophin in hypophysectomized rats has been demonstrated by Aron (6), Van Eck (154) and by Cortell and Rawson (32). In the intact, dynamic animal, however, an opposed reaction is the pituitary response to a relative lack of circulating thyroid hormone. All competent observers agree that in large doses thyroxine will lead to involution of the thyroid until a state is reached resembling that which follows removal of the pituitary. In this instance, the combined pituitary-hypothalamic effect must outweigh the local action on the thyroid's follicular cells.

#### F. ADMINISTRATION OF THYROTROPHIN TO MAN

On several occasions thyrotrophic preparations have been administered to human subjects. The classical observations of Thompson (148), however, showed that it was possible to evoke only a mild and transitory hyperthyroid state. Since the advent of  $\text{I}^{131}$  for tracer use, Stanley and Astwood (144) have re-examined the effect on the human gland of exogenous thyrotrophin. They used an Armour preparation from beef anterior pituitary glands containing 10 to 20 Waleszek-Koch chick units per  $\mu\text{g}.$  Single injections of 15 to 30 m. of this preparation were made in 23 normal subjects. Only after 8 hours could an effect be noted. At the end of this interval there was a marked acceleration of the rate of iodide uptake in the thyroid gland. This acceleration reached a peak in 24 to 48 hours, and did not return to the original level for nearly five days. If the activity of the human gland had been suppressed previously in consequence of thyroid medication, the effect of exogenous thyrotrophin was the more striking. Such single injections of thyrotrophin, although favoring the trapping of iodine, failed to produce a significant loss of total stored thyroid hormone. When the antithyroid drug mercaptoimidazole was also given, to prevent organic combination of the iodide, the same increase in rate of iodine uptake occurred. These observations suggest that in man enlargement of the iodinase (trapping) function of the thyroid acinar cells is an early effect of thyrotrophin. Although one usually associates the development of simple goiter with a relative lack of iodide, the experiments of Pal and Bose (107) indicate another and doubtless unique possibility. This is the hyperplasia induced in rats on an inadequate diet, but prevented by

adding *l*-tyrosine. Although of no significance in the etiology of human goiter, the finding is of fundamental interest, because it suggests that any deficiency leading to a lack of thyroid hormone will induce goiter, by increasing the secretion of thyrotrophin.

The effect of the removal or destruction of the normal thyroid upon non-functioning metastatic tumor tissue has been discussed by Rawson's colleagues (116) in a study of 21 patients. Eight of the metastatic solid and follicular adenocarcinomas became capable of fixing radioiodine within one and thirty-one months. It would appear that intense stimulation of metastatic thyroid cancers may alter their affinity for iodide. The clinical need for such an approach has been emphasized by Seidlin's colleagues (137) who found that among 14 unselected cases of *metastatic* thyroid carcinoma, a fixation of radioiodine could be demonstrated in only 8. Moreover, the intensity of the uptake was more closely related to the extent of preceding thyroidectomy than to the morphology of the tissue involved.

## II. Thyroid Blocking Agents

Various attempts have been made to diminish the net function of the thyroid gland without resorting to surgery (127). A generation ago Abderhalden and his associates attempted to solve this problem on the basis of specific organ-immunity (1). He supposed that the mammalian organism has a special mechanism for the destruction of foreign protein by a proteolytic mechanism. He found that the thyroid, among other tissues, could induce an antibody reaction when injected into normal animals; these animals when appropriately immunized, might produce enzymes which digested thyroid material. Somewhat later this approach was continued by Rogers and Beebe (123) who produced thyroimmune serum and actually used it in clinical cases. There seemed, indeed, to be some beneficial effect, but the problem was complicated by anaphylactic reactions some of which were fatal. Therefore, this line of approach has not turned out to be of great clinical significance, although there is an extensive literature on the subject of antihormones (75). The difficulty is, that in many so-called antihormone reactions the mechanism seems to be simply a reaction against the foreign protein supplied by another species of animal (159). Nevertheless, in the laboratory Ivy (103) was able to show that undeniable suppression of thyroid activity could be produced by such means. In recent years, Lerman (77) and Lerman and Meyer (98) have shown that species differences exist with respect to thyroglobulin antibodies. For example, the rabbit can be made myxedematous by such a technique whereas other species can not.

### A. DEPRESSION OF THE THYROID GLAND

It is convenient to divide the mechanisms of thyroid depression and the agents causing suppression of thyroid function into four chief categories. These are, respectively, suppression with

- (1) Thyroid hormone, including thyroxine and its derivatives.
- (2) Iodide, including (a) large doses of stable iodide and (b) a few millicuries of radioiodide.
- (3) Thyroid blocking agents, including (a) those goitrogens (like the thiocyanate ion) which interfere with iodide trapping by the iodonase enzyme and (b) those goitrogens (like propyl-thiouracil) which interfere with the periodase enzyme system.
- (4) Peripheral competitive agents which prevent the final action of the thyroid hormone in the general tissue cells; i.e., which block the action of "thyrenzyme."

In part, these agents involve the thyro-pituitary axis. In part, they operate directly upon the thyroid gland itself, as indicated in the section on thyrotrophin, immediately preceding. The effectiveness of radioiodine can be excluded from the present discussion; although the work of Skanse (142) suggests that a functional disability of thyroid enzymes may be produced by beta-rays without obvious morphological destruction—if the dosage of radioiodide be carefully controlled. The fact, however, that changes in thyroid histology can be demonstrated in hypophysectomized animals indicates that the thyroid gland itself is susceptible to stimulation other than that provided by the thyrotrophin. The experimental proof required to study this question is complicated by the divorce between function and histological appearance, which is so pronounced with thiouracil. No longer can the experimental pathologist hope to gauge thyroid function with a microscope!

### B. GOITROGENIC AGENTS

In recent years an important therapeutic approach has been made by Astwood (7) along chemotherapeutic lines. This approach grew out of the finding by Mackenzie, Mackenzie and McCollum (84) that the prolonged administration of sulfaguanidine to rats would produce goiter. Such goitrogenic effects could be prevented by giving appropriate doses of thyroxine, but not by the administration of iodide. The effects of this goitrogenic agent were studied in detail and perhaps the most striking finding was the fact that although the thyroid became markedly hyperplastic, nevertheless it failed to excrete enough hormone to keep the animal from myxedema. Indeed, as shown by Chaikoff (105), such goitrogens

may prevent the formation of diiodotyrosine or thyroxine both *in vitro* and *in vivo*.

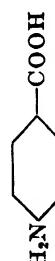
In retrospect, it appeared that other goitrogenic agents had similar effects. For example, the trials of prolonged thiocyanate therapy for hypertension had previously indicated that the internal economy of the thyroid gland could be interfered with. Clinical reports by Barker (155), by Means (112) and by O'Hare (122) had shown that such patients developed not only a prominent thyroid but also definite symptoms of hypothyroidism. The Dunedin (N.Z.) investigators, Kennedy and Purves (70) also studied rapeseed and its chemical goitrogenic agents, and reported similar changes in the gland. Following the observations of Barker and his colleagues (18) in man and the report by Mackenzie, Mackenzie and McCollum (84) that sulfaguanadine would produce goiter in rats, Astwood (7) initiated a systematic study of antithyroid drugs. Hundreds of compounds have already been tested in animals, and approximately half a dozen have had a considerable therapeutic trial. Unfortunately, the best drug for a rat is not always the best drug for a man, and *vice versa*. So far as is now known, the oxidative conversion of diiodotyrosine to thyroxine cannot be checked deliberately; although this process presumably involves an enzymic oxidation. Many such drugs appear to act upon the peroxidase enzyme system which converts the iodinase-trapped iodide to diiodotyrosine. Indeed Westerfeld and Lowe (160) have studied this reaction as catalyzed by peroxidase in the presence of hydrogen peroxide. Likewise, Keston (71) has shown that xanthine oxidase from milk can operate similarly. Why the pituitary gland is biologically essential, as shown in Fig. 1, remains unknown. This process obviously involves the transfer of electrons and is subject to the classical thermodynamic principles which govern oxidation-reduction reactions. In short, the fundamental laws of chemical affinity demand that the iodide ion, as supplied by the blood, be elevated to a state of oxidation equivalent to elementary iodine, or to hypoiodite. In addition to such thermodynamic considerations, however, certain other features influence the effectiveness of these drugs. In the most active group, the chief feature (presumably stereochemical) is the specific structure of the antithyroid drugs in question. In general, most of the known antithyroid drugs fall into two chief groups; the most active groups comprise thiourea and its congeners. The less active group, among which are the sulfonamides, contain an amino benzene nucleus as in 4, 4'-diaminodiphenylmethane and 4, 4'-diaminobenzil. In Table III are shown the comparative activities of some of these compounds. Many of these substances reduce elementary iodine very rapidly *in vitro* as pointed out by Miller, Roblin and Astwood (100). In so doing, the thio-compounds are oxidized to a cystine-like linkage. This theory, however, can be applied only to the first class mentioned, namely the thiocarbonamide derivatives.

The amino-benzene derivatives react only slowly with elementary iodine; and consequently no rational explanation of their action can be given at the present time. That the effect of an antithyroid may differ from thyroidectomy was shown by Leathem and Seeley (74) in studies with rats. In both instances there occurred rises in plasma non-protein nitrogen, globulin and total plasma protein concentrations. After thiouracil, however, the liver's weight increased and total liver protein increased, unlike the findings after thyroidectomy.

### C. GOITROGENS AND ENZYMES

The ultimate understanding of the behavior of the goitrogenic drugs will rest upon the elucidation of enzymic processes occurring within the thyroid acinar cells and perhaps also within the thyroid follicle itself. The follicular lacunae have long been regarded merely as storage spaces, rather than as chemical retorts. Their contents, however, arise as a protoplasmic budding-off, much as mucin ruptures from a goblet cell or milk globules from the lacteal parenchyma. These extruded droplets doubtless contain enzymes as well as thyroglobulin. The problem is to identify the enzymes, whether intracellular or intrafollicular. Obviously any enzyme system which is essential to fundamental metabolic processes is also needed for the formation of thyroxine. For instance, when the cytochrome system is poisoned by sulfide, cyanide or azide, thyroxine can not be synthesized as shown by Schachner, Franklin and Chaikoff (134). This finding, however, is not pertinent; because Lerner and Chaikoff (78) demonstrated that certain highly active antithyroid drugs failed to depress cell respiration. Indeed several derivatives of thiourea and several sulfonamides have been shown *not* to poison cytochrome oxidase or succinic dehydrogenase (149). Several investigators have suspected that inhibition of tyrosinase might explain antithyroid activity; especially because some goitrogens do inhibit this enzyme. There is no good evidence, however, that tyrosinase occurs in the thyroid parenchyma. Furthermore, Dubois and Erway (42) found that several derivatives of thiourea showed more predilection for general toxicity than for antithyroid effects, as judged from their inhibitory effect on tyrosinase. A search for the specific effects of heavy-metal complexes, suggested by Reineke and Turner (117), has likewise proved fruitless. Other enzyme systems have been studied, notably peroxidase, which might expedite two essential stages in the synthesis of thyroxine, i.e., the formation of diiodotyrosine and the conjugation of two such molecules or radicles. Quite possibly the latter reaction occurs after the colloidal framework of thyroglobulin has been laid down. Thyroid cells do contain such an enzyme, as shown by Dempsey (33). Unfortunately, although several goitrogens inhibit peroxidase, De Robertis and Grasso (37) found that sulfonamides fail to do so. One is left, therefore, merely

TABLE III  
CHEMICAL TYPES OF THYROID BLOCKING AGENTS AND THEIR ACTIVITY (IN RATS)<sup>a</sup>

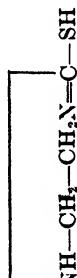
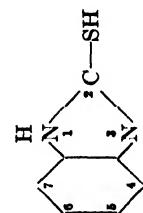
Compound	Formula	No. of animals	Ave. dose in food %	Av. dose mg./kg./day	Body weight gain g./day	Thyroid weight mg./100 g. rat	Thyroid iodine mg.-%	Estimated activity thiouracil = 100
<i>Derivatives of Thiourea</i>								
Thiourea	$\text{NH}_2\text{CNH}_2$ 	3	0.01	12	3.6	8.8	36.7	9
		3	0.05	58	3.4	18.0	16.2	
		3	0.10	108	2.6	34.4	2.7	
		3	0.50	420	1.5	26.3	2.3	
Phenylthiourea	$\text{C}_6\text{H}_5\text{NHCSNH}_2$	6	0.01	11	1.9	8.3	31.7	
		6	0.10	62	-4.0	21.2	8.5	14
<i>Derivatives of Aniline</i>								
Pyridine-2-thiol	$2-\text{C}_5\text{H}_4\text{NSH}$	3	0.01	10	3.4	9.9	25.8	
		3	0.03	29	2.7	18.4	3.0	
		3	0.10	82	1.0	27.2	4.3	
<i>p</i> -Aminobenzoic acid		3	0.01	11	3.7	6.3	65.9	
		3	0.10	110	3.8	5.5	70.7	
		3	1.0	1190	3.7	8.2	22.2	
		3	5.0	3980	0.8	23.5	4.9	

<b>2-Thiazoline-2-thiol (2-mercaptopthiazole- line)</b>		3	0.0003	0.3	3.3	5.9	46.4	131						
		3	0.006	0.6	3.2	7.5	39.6							
		6	0.001	1.1	3.3	7.6	36.3							
		3	0.003	3.0	3.4	8.1	19.4							
		3	0.005	5.5	2.8	13.9	7.2							
		3	0.006	5.8	3.3	14.1	4.4							
		6	0.01	10.0	3.4	25.2	2.8							
		3	0.03	30.0	3.4	24.6								
		3	0.06	54	2.0	30.2	1.4							
		6	0.10	78	1.7	31.2	2.9							
<b>Cyanides and Thiocyanates</b>	KSCN													
Potassium thiocyanate														
<b>Pyrimidines and Thio-barbituric Acids</b>														
<b>4-Methyl-6-hydroxy- 2-mercaptopurimi- dine (4-methylthio- uracil)</b>		6	0.01	12	3.9	17.4	3.0	104						
		6	0.10	105	2.6	30.6	1.9							
<b>5,5-Diethyl-2-thio- barbituric acid (thiobarbital)</b>		3	0.0006	0.6	2.2	7.1	39.4							
		3	0.001	1.2	2.9	6.9	33.1							
		3	0.003	3.1	2.7	7.9	17.9							
		3	0.006	6.3	2.8	14.5	5.9							
		3	0.01	10	2.7	17.7	4.7							
		3	0.03	33	3.0	18.1								
		3	0.06	63	2.6	21.9	1.5							
		3	0.10	108	2.9	20.5	2.1							
		3	0.3	300	2.5	23.5	1.9							

++++

On low iodine intake

TABLE III (*Continued*)

Compound	Formula	No. of animals	Conc. in food %	Av. dose mg./kg./day	Body weight gain g./day	Thyroid weight mg./100 g. rat	Thyroid iodine mg. %	Estimated activity thiouracil = 100
<i>Imidazoles* and Benzimidazoles</i>								
2-Imidazole-2-thiol		3 6	0.01 0.10	11 73	3.3 -0.7	9.2 18.1	11.3 8.5	63
Benzimidazole-2-thiol		6 3 6 3 3 3	0.0003 0.0006 0.001 0.003 0.006 0.006	0.3 0.7 1.1 3.3 6.4 11	3.2 2.8 3.1 3.0 3.3 3.2	6.5 7.3 8.7 10.7 11.1 23.1	51.3 39.4 36.1 14.9 13.0 3.6	116

<i>Sulfones and Sulfides</i>								
4-Aminophenyl-2'- amino-5'-thiazolyl sulfone (promizole)		3	0.001	1.2	4.1	7.6	43.5	18
		3	0.003	3.4	3.5	8.5	28.4	
		3	0.006	7.1	3.6	8.0	28.8	
		6	0.01	13	3.7	9.6	27.2	
		3	0.03	36	3.8	11.3	9.3	
		3	0.06	75	3.8	24.4	2.0	
		6	0.10	109	2.9	29.0	2.1	
		3	0.30	300	1.8	34.2	2.8	
		3	0.60	650	0.4	40.7	1.8	
		3	1.0	1000	1.0	18.6	9.8	
4-Aminophenyl-2'- amino-5'-thiazolyl sulfide		3	0.0006	0.7	3.3	5.1	40.3	53
		3	0.001	1.2	3.3	5.9	45.7	
		3	0.003	3.6	3.4	7.7	35.2	
		3	0.006	6.6	3.3	7.8	22.1	
		9	0.01	11	3.1	9.3	11.3	
		3	0.03	35	2.7	15.5	2.1	
		6	0.055	55	2.6	23.7	1.7	
		9	0.10	96	2.4	28.0	1.8	
		3	0.3	284	1.4	38.2	1.4	
		3	0.50	398	1.0	29.9	1.9	
		3	1.0	532	-0.3	23.3	4.6	

<sup>a</sup> Compiled from excerpts of data from McGinty (92), and Astwood (8) and their associates.

<sup>b</sup> Recently Stanley and Astwood have found that 1-methyl-2-mercaptopimidazole in man is a hundred times as active as thiouracil. (*Endocrinology* 44, 49 (1949).)

with speculations as to the role of the antithyroid drugs: Do they inhibit the periodase system directly or compete with iodide for the capture of enzyme molecules? Do they reduce elementary iodine (or its equivalent) as fast as it is produced? Do they preferentially steal the hydrogen peroxide required for the oxidation of iodide to a state of higher oxidation? No one knows. That enzymic action can result in the synthesis of thyroxine was demonstrated by Keston (71), who added radioactive iodide and xanthine to milk and recovered radioactive thyroxine. In this instance the xanthine oxidase of milk yielded the hydrogen peroxide upon which the peroxidase of the milk could act. It is significant that in the presence of thiourea, no thyroxine could be found. That methylthiouracil has a general effect on tissue respiration was shown in thyroidectomized rats treated with methylthiouracil. Borell and Holmgren (24) found that tissue respiration fell lower than a mere lack of thyroid hormone could explain. The thyroid was an exception, because respiration increased and the acinar cells underwent hyperplasia. When 0.2% thiouracil was included in the feed of pregnant mice, Kauffman, Hurst and Turner (67) found that the fetal thyroids were affected. Normally follicle and colloid formation begin on 16th day of mouse fetal life. Presumably because thiouracil passes through the placenta, the formation of thyroid follicles is inhibited and the formation of colloid depressed.

Further studies by Richter and Clisby (119) and by Kennedy (69) showed that thiourea derivatives also produced a goitrogenic effect. On histological examination of the tissues of animals so treated, the thyroid displayed very small follicles, irregular in shape and empty of colloid. The follicular cells were high columnar in form, with large round nucleii and frequent mitoses. After continued administration of the drug, the follicular cells underwent such hyperplasia as almost to fill completely the lumen of each follicle. After these observations, many other derivatives were studied. It appears now that a large variety of substances can act as goitrogens (9). In fact, already over 200 compounds have been evaluated in animals (8). Among the sulfur-containing compounds found to be useful were 2-thiouracil, 2-thiobarbituric acid and diethylthiourea. A large number of other derivatives might be mentioned in the same category: for example, *sym*-diethylthiourea; 5-benzal-2-thiohydantoin; 5,5-diethyl-2-thiobarbituric acid; pyridine-2-thiol; thiazoline-2-thiol and benzimidazole-2-thiol (92). In rats these drugs effectively block the synthesis of thyroxine.

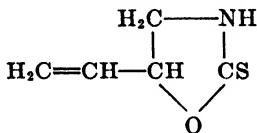
Other groups of compounds have also been studied. These comprise such aniline derivatives as sulfaguanidine; the *p*-, *m*-, and *o*-amino benzoic acids; *p*-aminoacetanilide and *p*-aminophenylacetic acid. Several major studies of various derivatives of these compounds are still under way and

doubtless other drugs useful in therapy will be discovered (94). Such compounds as thiadiazole-2-thiol, for example, merit further study.

As pointed out in 1945 by Salter, Cortell and McKay (132) iodide differentiates between the effects of thiocyanate and of thiouracil, respectively, upon hyperplasia of the thyroid. These investigators took the precaution of producing a mild goiter (in the rat) before testing the further effect of these different goitrogens. Other studies by Mackenzie (83) have shown that small amounts of iodide inhibit the goitrogenic action of thiouracil by 50 to 100 per cent, depending upon the dosage of goitrogen. At high levels of iodide intake, the thyroid hyperplasia is still suppressed; but the effect fails when sublethal doses of thiouracil are used. Mackenzie found that when a low dosage of sulfaguanidine is combined with a high dosage of iodide, they exhibit augmentation. The synergistic effect fails, however, when iodide intake is low or sulfaguanidine intake is high. The degree of hyperplasia produced by these drugs, however, was invariably increased by iodide under all combinations of dosage. Thus, sulfaguanidine appears to differ from thiouracil, thiourea and *p*-aminobenzoic acid in that the latter group's goitrogenic and hyperplastic effects are *not* both inhibited by iodide. In such glands, Salter, Cortell and McKay (132) found that the ability to trap iodide remained; and this was also found by McGinty and Sharp (93) and by Astwood (10). In synopsis, it must be confessed that when bizarre extremes of dosage are used, the qualitative distinction between various goitrogens falls down. In other words, at extreme dosage, probably they all can inhibit both the iodinase and periodase systems, as described earlier. When large doses of thiouracil are administered to rats, a persistent failure to trap iodine results. In part, this deficiency can be overcome by large doses of iodide. In this respect thiocyanate seems to differ from thiouracil both in the qualitative response and in the quantitative aspects of the dosages used. In the presence of either drug large doses of iodide will facilitate the storage of iodine; but conversion of iodide to diiodotyrosine (and incidentally to thyroxine) is prevented. The synthesis of uniodinated thyroid protein and cellular hyperplasia can proceed unimpaired despite heavy dosage of thiouracil. Hyperplasia under thiocyanate, however, is nullified by iodide.

As mentioned earlier, it is convenient to assume that all diets are at least mildly goitrogenic. Moreover, the normal saliva contains a characteristic concentration of thiocyanate ion; so that it is even conceivable that a goitrogenic effect is produced in the course of normal metabolism. From this standpoint, the striking goiters produced in rabbits on a cabbage diet by Chesney, Clawson and Webster (29) might be regarded as merely an extreme manifestation of a normal trend. If one accepts this point of view, all animals are continuously subjected to goitrogenic substances.

Many foods have been shown to have goitrogenic activity. As early as 1800 Barton of Philadelphia (22) had called attention to goiters in Tyroleans who ate a variety of Alpine chestnuts. In addition to von Suk's observations on oats and Chesney's on cabbage, Hercus (62) has described as goitrogenic chard, rape, and turnips; Sharples (140) has named soybeans; and McCarrison (89) peanuts. Recently Greer and Astwood (59) have studied the effect of 61 different foods on man, with the help of tracer iodide. The most goitrogenic of these foods was the rutabaga, the active principle of which could be extracted from an aqueous solution with ether. Animal products were not very active; but cow's milk, beef liver, and oysters had some effect. Among the most potent vegetable families were the following: Chenopodiaceae, Compositae, Cruciferae, Cupuliferae, Juglandaceae, Leguminosae, Rosaceae and Umbelliferae. These include such homely items as spinach, turnip, honeydew melon, walnut, lima beans, peas, pears, strawberries, grapefruit, carrots, and grapes. The pure goitrogenic substance was isolated in crystalline form from the yellow turnip. It proved to have the formula: *l*-5-vinyl-2-thioxazolidone, i.e.,



and was detected not only in the roots of white turnips but also in the seeds of white and yellow turnips, kale, rape and cabbage.

Studies of the behavior of iodide, concentrated in the thyroid glands of young rats while under the influence of propylthiouracil, have been reported by Vanderlaan and Vanderlaan (155). Both chemical analyses and measurements of tracer and radioactivity gave the same results. The iodine so held is entirely in the reduced (i.e., iodide) form, when titrated potentiometrically with a silver-silver iodide electrode. It is entirely ultrafilterable. Thus a gradient exists between the serum- and thyroid-iodide concentrations unless the serum iodide is relatively concentrated. With propylthiouracil, this gradient increases some tenfold as thyroid hyperplasia ensues. Consequently the total enlarged mass of the gland can fix some thirty times the amount of iodide fixed by control glands under similar circumstances. When thiocyanate is administered, it inhibits the preferential concentration of iodide and also causes a discharge of previously concentrated iodide. A mathematical description of this phenomenon has been given by Salter (130). It is interesting that neither bromide nor sulfaguanidine exhibit a comparable effect. The interrelation of the goitrogenic effect with iodine has been applied by McGinty and his colleagues in order to assay various probable antithyroid drugs. As shown in Figs. 8 and 9 the iodine concentration within the gland is

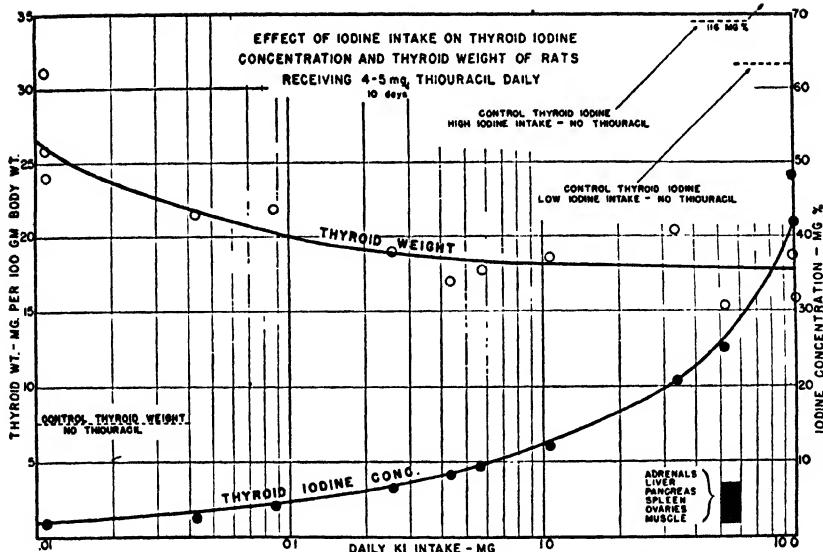


FIG. 8.—From McGinty, Iodine Absorption and Utilization, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 405, 1949.

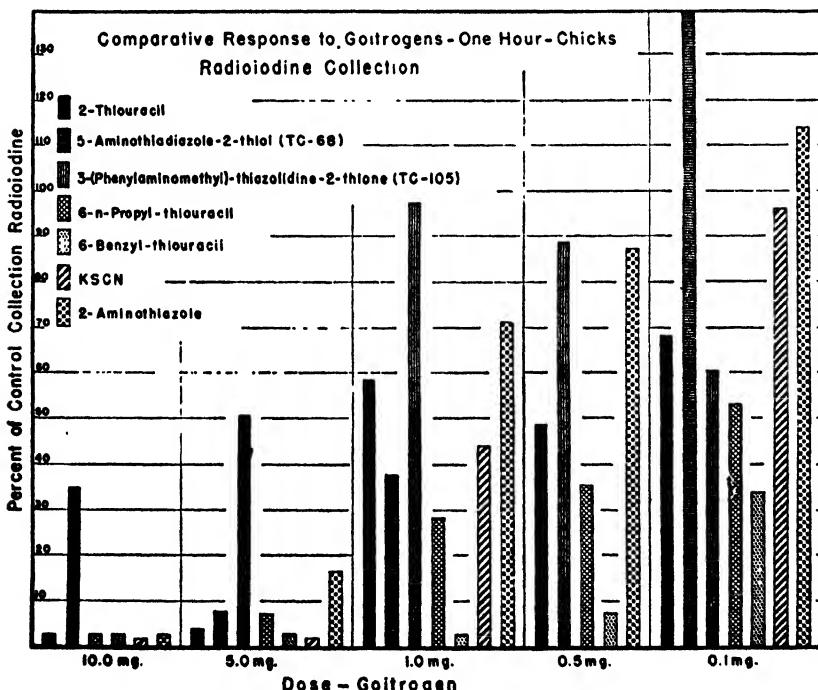


FIG. 9.—From McGinty, Iodine Absorption and Utilization, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* 50, 417, 1949.

markedly affected by thiouracil or by other goitrogens. Both rats and chicks have been studied with radioactive iodide by this method. The effect of several goitrogens on the rat thyroid is shown in Fig. 10.

It can be demonstrated readily that the thiocyanate ion and the iodide ion are in competition as described in the preceding section. The clinical significance of this was elucidated by Barker and his colleagues (18), who

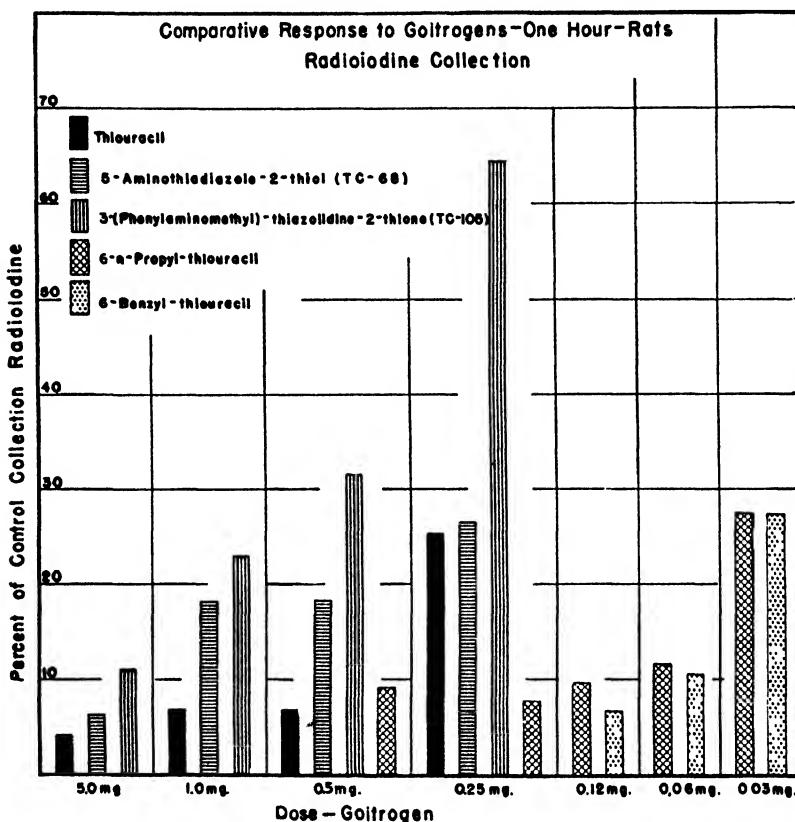


FIG. 10.—From McGinty, Iodine Absorption and Utilization, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* **50**, 416, 1949.

showed that the resulting hypothyroidism in hypertensive patients could be relieved by administering one or two grains of desiccated thyroid daily. The severe myxedema of such patients has been described also by Rawson, Hertz and Means (112). Working with animals, Astwood (8) found that the goitrogenic effect could be counteracted by increasing the iodide content of the diet. Moreover, studies of slices of thyroid tissue *in vitro* by Franklin, Chaikoff and Lerner (50) showed that thiocyanate prevented the

uptake of iodide from the medium. In brief, the initial trapping of iodide is prevented by the presence of thiocyanate. As suggested in the preceding section the experiments of Salter, Cortell and McKay (132) indicate that an easily reversible complex is formed with a thyroidal protein (iodinase).

TABLE IV  
IODINE FRACTIONS IN FRESH THYROIDS OF MALE RATS TREATED WITH GOITROGENS<sup>a</sup>

Rat No.	Wt. of rat, gm.	Wt. of thy- roid, mg.	Fraction of homogenate	Iodine in thyroid ( $\mu\text{g.}$ )			
				Total iodine	After dialy- sis	Heat co- agu- lable	Ppt'd by acetone
14 B Control	270 to 327 <sup>b</sup>	32.0	Protein Iodide		0.3	0.7 2.5	0 3.0
			Sum	2.7		3.2	3.0
21 G Thiouracil plus KI	220 to 296	167.1	Protein Iodide		2.0	2.7 8.3	4.7 6.9
			Sum	11.5		11.0	11.6
25 G Thiouracil plus KI	250 to 383	298.5	Protein Iodide		3.2	3.0 9.6	6.1 7.9
			Sum	14.0		12.6	14.0
22 E Thiocyanate plus KI	290 to 376	35.9	Protein Iodide		1.5	3.5 2.4	0 4.1
			Sum	3.3		5.9	4.1

<sup>a</sup> From Salter, W. T. "Metabolic Circuit of the Thyroid Hormone," in "Thyroid Function as Disclosed by Newer Methods of Study." *Ann. N. Y. Acad Sci.* **50**, 368 (1949).

<sup>b</sup> Weights are given for the beginning and the end of the 9-month period during which the animals were under study.

This can be precipitated from extracts of the gland (especially if hyperplastic) with cold acetone, as shown in Table IV. If this protein is heat-coagulated, however, it gives up its iodide. This colloidal complex serves to fix ions as they diffuse through the cell membranes. Because of the large size of the colloidal complex the bound iodide cannot diffuse back into the extracellular fluid. Because this iodide fraction,  $I_B$ , is in direct equilibrium with free iodide ions, it behaves thermodynamically like

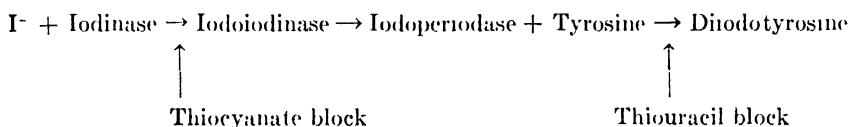
iodide in systems which permit reversible equilibria, as shown by Vanderlaan and VanderLaan (152). Through this mechanism, the thyroid gland is able to accumulate iodide at concentrations well above tenfold that of the blood stream. Indeed, in hyperplastic glands the concentration gradient may exceed 200-fold under exceptional circumstances. When animals are treated with thiocyanate a prompt release of this reversibly trapped iodide occurs. Probably the organic cyanides (such as nitriles, oil of mustard, cyanoglucosides) as well as isothiocyanates and the free thiocyanate occurring in plants have similar effects. This interpretation would apply likewise to the production of goiter by such plants as the Umbelliferae and the Brassica group.

Because thiouracil proved so effective a thyroid blocking agent, albeit somewhat toxic, many derivatives of this prototype have been studied (14,95,96). For example, studies by Stanley and Astwood indicate that the activity of 1-methyl-2-mercaptoimidazole *in man* is a hundred times that of thiouracil.

Comparative clinical studies are available (90) for the effectiveness of methyl- and propylthiouracil in the treatment of hyperthyroidism. In doses of 200 to 400 mg. daily the methyl compound appears to be slightly more effective than the propyl derivative. Some observers think it slightly more toxic. With respect to the prolonged use of methylthiouracil in thyrotoxicosis, Meulengracht and Kjerulf-Jensen (97) studied 190 patients treated with this drug which they consider better than propylthiouracil. In 13 of these patients, "hypersensitivity" led to the abandonment of the drug. They concluded that prolonged treatment with methylthiouracil counteracts thyrotoxicosis and shortens the duration of the disease. Although 9% of their cases relapsed, the recurrent thyrotoxicosis responded promptly to resumed therapy. The case for the propyl derivative has been stated by Reveno (118), who controlled thyrotoxicosis in 95 patients, of whom 67 had toxic nodular goiter and 28 toxic diffuse hyperplasia. Among these he also has discussed 12 other reports which can not be reviewed here. Only Wilson and Goodwin (163) preferred the methyl derivative to the propyl. In general, remission followed a daily dose of 150 to 250 mg. within two months. The effect appeared somewhat more slowly than with thiouracil. It was not greatly impeded by the previous administration of iodine. Only one case of agranulocytosis had been reported (72) (20). Reveno noted no intolerance during prolonged therapy on a maintenance dose of some 90 mg. daily. Three patients were safely treated throughout pregnancy and delivered of normal babies. Bartels (21) has summarized five years of clinical experience with the use of antithyroid drugs. He states that the effective doses of thiouracil, propylthiouracil and methylthiouracil, respectively, are 600 mg., 200 to 300 mg., and 200 to 300 mg. The respective incidences for toxic reactions per 100 patients are 9, 2 and 10. The original enthusiasm for these drugs as complete substitutes for surgery has been modified by certain difficulties. For instance, although mild thyrotoxicosis may undergo a prolonged remission after a course of treatment, cases with severe "toxicity" and large glands require combined therapy to control them. In adenomatous goiters, prolonged treatment is not suitable.

#### D. DISTINCTION BETWEEN THIOCYANATE AND THIOURACIL

Working with rats, Salter, Cortell and McKay (132) studied the possible antagonism of iodide against thiocyanate and thiouracil, respectively. Heavy doses of iodide prevented the goitrogenic effect of thiocyanate. Nevertheless the glands treated with thiouracil were able to fix (in reversible combination) a high concentration of iodide. Other observations indicated that the thiocyanate inhibited the action of iodoiodinase, whereas the thiouracil inhibited the action of periodase. This interpretation can readily be understood in terms of the following classical scheme for the natural synthesis of thyroxine:



These two respective drugs produce "bottlenecks" in the natural chain of synthetic reactions at different sites. In either case, however, the formation of hormone is interfered with. The blockade by thiocyanate can be "forced" if an extra supply of iodide becomes available. These results on animals have been confirmed in human individuals by Astwood and Stanley (16). Thiouracil produced a gradual diminution in thyroidal iodine. On the contrary, thiocyanate caused a prompt release of radioactive tracer from the human gland. These observations with tracer iodide on the effect of thiocyanate have been extended by Stanley and Astwood (143) to patients suffering from thyrotoxicosis after treatment with thiouracil. Even after the antithyroid drug had checked practically all organic combination, the goiter was able to fix radioiodide. Moreover the newly trapped iodide could be discharged either by administering thiocyanate or a large dose of stable iodide. The patients with Graves' Disease fixed more labeled iodide than did normal patients presumably because their acinar cells and follicles had enlarged. If the gland were not sufficiently poisoned with thiouracil to prevent organic combination, the labeled iodine so combined (chiefly as diiodotyrosine) was not discharged by thiocyanate or iodide in heavy dosage. This feature can be used for a test of adequate dosage with antithyroid drugs.

#### E. CHEMICAL CYTOLOGY AND ANTITHYROID DRUGS

Studies by Dempsey and Astwood (34) have revealed characteristic changes in the chemocytology of the thyroid under various physiological conditions. For example, exposure of animals to cold doubles the rate of secretion, as shown in Fig. 11, Nos. 10 and 11 (Pap's stain, rat).

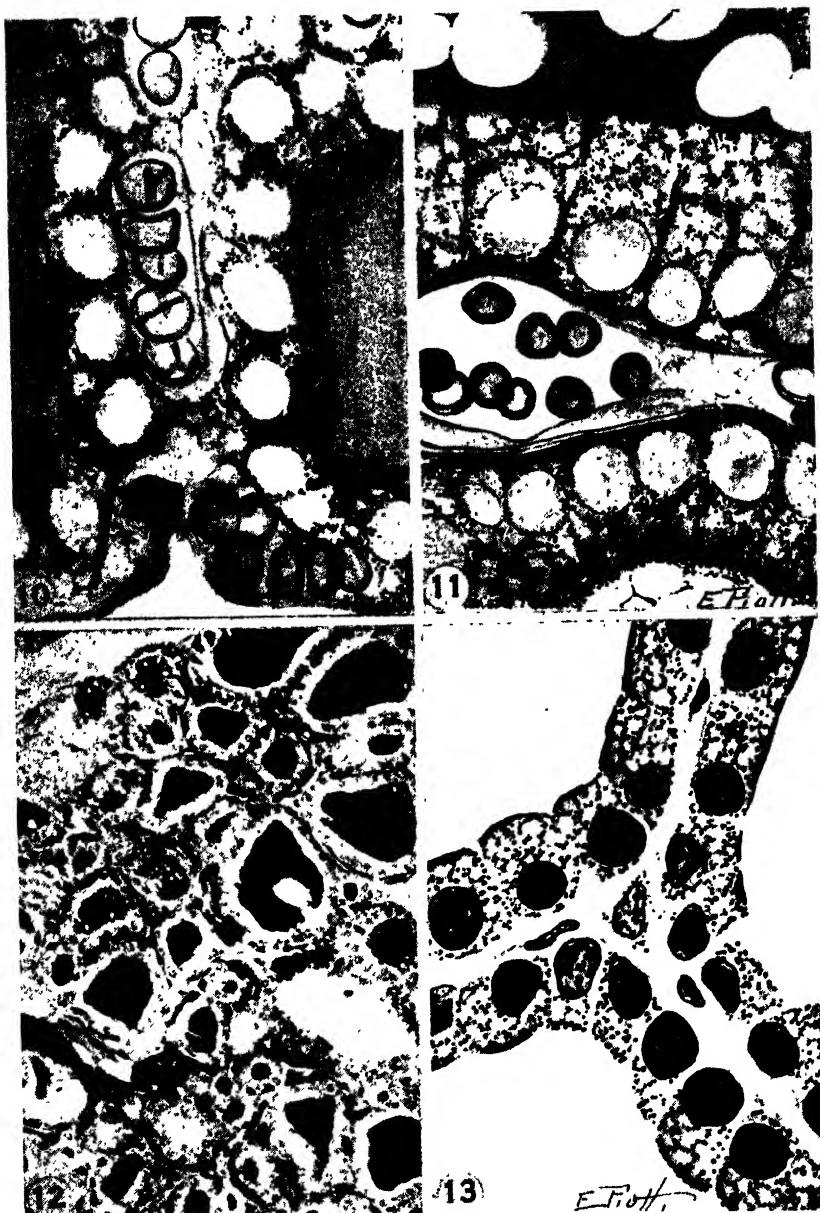


FIG. 11.—Cytoplasmic granules in thyroid cells (cf. text). From Dempsey, E. W. *Ann. N. Y. Acad. Sci.* **50**, 348, 1949.

During increased release of hormone, while the height of the follicular cells increases, the concentration of nucleoproteins in the colloid decreases and

in consequence its basophilia. At the same time the nuclear desoxyribonuclear proteins in the follicular cells decline slightly and the sudanophilic droplets increase. During active internal secretion, the colloid loses reducing substances and its autofluorescence falls. Meantime, the alkaline phosphatase decreases in the capillary endothelium, while acid glycero-phosphatase appears in the acinar cells. When poisoned with thiouracil, the only difference from the preceding reaction is that the endothelial phosphatase is increased when synthesis of the hormone is blocked. When the colloid has been exhausted by the continuous influence of thiouracil, hypophysectomy may be performed. Then, whether the antithyroid drug is stopped or not, the colloid is re-formed; but it contains practically no iodine. The general picture of these glands resembles that of animals kept in a warm environment. The epithelium is low cuboidal, the cells and colloid become intensely basophilic and the reducing activity of the colloid is high. The only difference is that the poisoned glands show no phosphatase in the capillaries. Thus both nucleoproteins and reducing substances can be formed in the absence of the pituitary during thiouracil poisoning. The cellular hypertrophy and hyperplasia, however, which occur under the influence of thiouracil are dependent upon the thyrotrophin. Carbonyl groups are shown in No. 12 (leucofuchsin, normal rat) and argyrophilia in No. 13 (normal dog).

#### F. GROSS PHYSIOLOGIC EFFECTS

Astwood and his group (7) and Himsworth (65) were among the first to study the application of these substances in clinical cases. Thousands of cases have now been accumulated all over the world (88,102) and certainly in many instances the effect in the clinic is striking. The basal metabolic rate fails, body weight rises, the concentration of blood cholesterol increases; and, what is most important, the clinical symptomatology of hyperthyroidism all but disappears in successfully treated cases. In experimental animals a decline of the normal metabolism can be observed. For example, Astwood (10) administered sulfaguanidine to young rats and followed the food consumption, growth rate, and development of the animal. Presently, in spite of the development of a goiter, the animals ate less food, grew less rapidly than controls, and showed other manifestations of hypothyroidism. When thyroid hormone was then administered, these effects were abolished, and the goitrous effect of the drug was checked. Moreover, in hypophysectomized animals no enlargement of the thyroid occurred,—a fact which indicated that the thyrotrophic hormone was the immediate agent which produced the hypertrophy of the thyroid. When the goitrogen and thyroid hormone were administered simultaneously, the goitrogen had no influence on the calorigenic or toxic effects of the thyroid hormone. This was true in normal, in hypophysectomized, and in thyroi-

dectomized animals alike. Consequently it seems clear that the primary action of this group of drugs is an inhibition of the formation of thyroid hormone. As shown in Fig. 12, young rats exhibit a nearly complete loss of iodine from the thyroid gland soon after treatment is begun. No new hormone can be formed, and one-half of the original store in the gland is used up in a day. The result is that all of the thyroid reserve is exhausted in five days. Simultaneously, the circulating hormone decreases (82), and, in consequence, the thyroid gland is stimulated to compensatory enlargement by virtue of pituitary thyrotropic activity. Under these

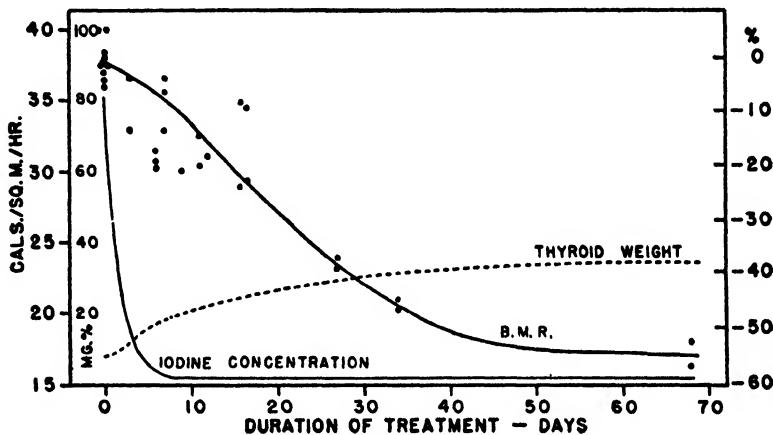


FIG. 12.—Effects of the administration of thiouracil in a concentration of 0.1% of the diet on the iodine concentration and weight of the thyroid gland and on the basal metabolic rate of young rats. The scales on the left and right refer to calories per square meter of body surface per hour and the percentage change in metabolic rate respectively. The inner scale on the left refers to the iodine concentration in mg. per 100 g. of wet tissue and the thyroid weight in mg. per 100 g. body weight. (From Astwood, *Harvey Lectures XL*, 197, 1944-45.)

conditions, within two months, the basal metabolic rate has declined to levels lower than those ordinarily encountered after thyroidectomy. If the administration of the blocking agent be prolonged, the rats fail to develop and grow. Indeed, when rats are treated from birth, definite cretinism results: although this can be prevented if an adequate excess of thyroxine be administered simultaneously to control animals.

#### G. SPECIES DIFFERENCE

One of the interesting complications of these studies is the fact that there is considerable difference among species, among animals of different strains (101), and even among animals of different ages in the same strain (129). Young animals seem to be particularly susceptible to goitrogens. In the

case of sulfaguanidine, for example, guinea pigs exhibit very little response, whereas rats and chicks show marked effects (84).

Some curious species differences are encountered in the comparative response to thiouracil. For example, the guinea pig responds less markedly than the rat,—especially true in very young animals. Likewise the mouse seems to be quite insensitive to the compound (156). In the Rhesus monkey only a slight effect is noted (46). Chicks treated with thiouracil, however, show a tremendous enlargement of their thyroids so that these little creatures resemble tiny ostriches, each swallowing an orange. The body feathers, comb, wattles, and spurs remain undeveloped. The joints are hypermobile, the muscles weak and the body skeleton much retarded in development. Ultimately, the obese chick is unable to stand. These goitrogenic drugs are also effective in lower forms of life. Normal amphibian metamorphosis is checked in certain species (58), notably in *Rana pipiens*. Likewise the environment may affect the result: for example, thyroid hyperplasia in rats treated with thiouracil practically disappears in a sufficiently warm environment. Some goitrogens are effective only when a low iodine intake prevails, as with methylecyanide and thiocyanate; whereas thiourea and certain aminobenzenes are effective even in the presence of a rather high iodine intake. Possibly this difference is merely one of degree rather than of mechanism. Indeed, Salter (132) has suggested that there is some competitive action between the iodide concentration and the concentration of the goitrogen within the gland as it passes from the circulating blood.

### B. ASSAY OF THYROID ACTION

Astwood and his collaborators (14) have utilized the goitrogenic response as a means of assaying samples of thyroid hormone or of thyroxine. When animals are given a known dose of a goitrogen, the response of their thyroids in terms of hyperplasia can be measured, e.g., by crude weight. One can then determine in control animals how much goitrogen is required to nullify the effect of a certain amount of thyroxine or a related substance. Thus, there is a sort of reciprocal relationship between the amount of thyroid hormone available and the amount of goitrogen being used. This balancing of two drugs one against the other is an interesting and ingenious method of bioassay, which may prove useful when only small amounts of material are available. The method is illustrated by the accompanying figure, taken from Turner (135). Indeed, it is possible by this method to distinguish between optical isomers of thyroxine (Fig. 13).

### I. MODE OF ACTION OF ANTITHYROID SUBSTANCES

The actual mode of action of thiouracil is not as yet fully understood but the drug is stored in the gland (13), and this storage is decreased by

thyrotrophic hormone and increased by potassium iodide. The mechanism involved is uncertain, but Werner (158) and Astwood's associates have demonstrated that *in vitro* thiourea and thiouracil cause reduction of free iodine to iodide or its equivalent. Incidentally, Dempsey (33) studied a peroxidase in thyroid cells and presented evidence that it tends to disappear when thiourea is administered. It is obviously suggestive that the formation of thyroid protein may continue independently of iodine metabolism as pointed out by Salter (132).

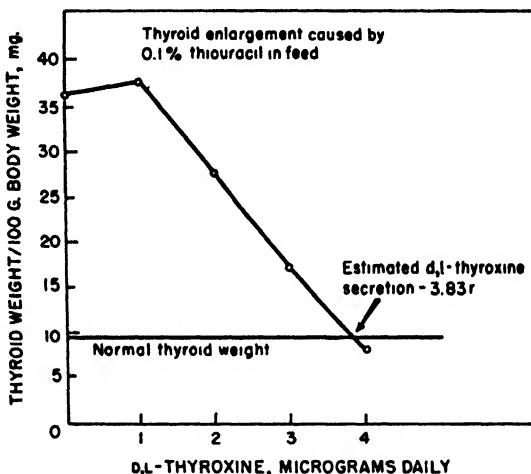


FIG. 13.—Normal rate of thyroxine secretion in fowl. The weight of the thyroid was determined in 4-week-old, thiouracil fed White Leghorn cockerels. In addition racemic thyroxine was administered, and this medication counteracted, in whole or in part, the failure of the thyroid gland to produce its own. When the normal thyroid weight was added, it was assumed that the investigator had given a daily dose equivalent to the normal daily secretion by the gland. (From Schultze and Turner, *Yale J. Biol. and Med.* 17, 273, 1944.)

Further studies have been made on the mechanism of these drugs with respect to cellular mechanism. Williams and his associates (161) showed that the anatomical changes in other organs were insignificant. Rawson, Tannenheimer and Peacock (73,114) observed in rats that the uptake of tracer doses of radioactive iodine by the thyroid was markedly diminished by thiouracil, whereas after thiocyanate the uptake was increased. Similar findings were reported also by Franklin, Lerner and Chaikoff (50) who showed also that these drugs prevented the fixation of iodine by thyroid tissue *in vitro*. The mechanism of this group of drugs has been pursued further by Salter and his associates (132) who fractionated the thyroxine-like and diiodotyrosine-like fractions from the inorganic iodide fraction in glands. They found that when large doses of thiouracil were administered

to rats the persistent failure to trap iodine could be overcome in part by large doses of iodide. In this respect, therefore, thiocyanate seemed to differ from thiouracil both in the qualitative response and in the quantitative response. Either of these drugs would check the storage of iodine, but the administration of larger doses of iodide would produce further iodine storage. The superficial explanation of their action is that the conversion of iodide to thyroxine is prevented. The synthesis of uniodinated protein, however, can proceed unimpeded despite heavy doses of thiouracil; and, at the same time, marked hyperplasia of the gland progresses even though heavy dosage of iodide is given. Hyperplasia under thiocyanate, however, is nullified by moderate doses of iodide, whereas it proceeds with little (if any) impairment under the chronic influence of thiouracil, when given in heavy dosage.

When the diiodotyrosine-like and thyroxine-like fractions were studied in these glands (132), it was found that with thiouracil practically no diiodotyrosine was formed and virtually no thyroxine. Under thiocyanate, however, a considerable amount of diiodotyrosine was produced, but only a little thyroxine accumulated. Perhaps the most striking difference between these two drugs was the effect of superimposing large dosages of iodide. In the case of thiouracil a large amount of this iodide was trapped in the form of a labile complex which may be interpreted as the first step in an enzyme reaction. This enzymic complex, however, was unable to transfer the iodine to the tyrosine of the protein. Therefore a "bottleneck" was formed between the "I" and the "D" Stage of the classical series of biosynthetic reactions. On the contrary, with thiocyanate a bottleneck appeared after the "D" stage, so that little iodide accumulated but a considerable amount of diiodotyrosine. As might be expected, in this case very little thyroxine-like material accumulated because the "bottleneck" in this instance followed the "D" stage. These results have been amplified and extended in recent investigations from several laboratories. Through the courtesy of Drs. R. W. Rawson and D.A. McGinty some of these findings are given in the following paragraph.

Rawson and McGinty (113) compared, in the chick and the rat, both the acute and chronic effects of several drugs. Chief among these compounds were potassium thiocyanate, thiouracil, TC-68 and TC-105. The respective effects can be summarized as follows:

	Chick	Rat
<i>Acute Effect</i>		
KSCN.....	Blocks for 6 hours	Blocks for 6 hours
Thiouracil.....	Blocks for 6 hours	.
TC-68.....	Blocks for 6 hours	.
TC-105.....	Blocks for 6 hours	.

	Chick	Rat
<b>Chronic Goiter</b>		
KSCN.....	.....	Increases
Thiouracil.....	Blocks	Blocks
TC-68 .....	Increases	Blocks
TC-105.....	Increases	Blocks

The effects were studied by means of radioiodine and varying results were observed. All of these agents when administered acutely by injection produced a block to the progress of iodine metabolism up to 6 hours after injection of the drug. Rats made goitrous by these agents collected less iodine except in the case of KSCN. Animals made goitrous with this latter agent collected much more iodine in their thyroids than did the controls. Chicks, on the other hand, made goitrous with thiouracil and TC-68 and TC-105 handled the injected tracer iodine differently. Thiouracil goiters failed to trap the iodine whereas the goiters produced by the other two agents collected more iodine than did the controls. Compound TC-68 is 5-aminothiadiazole-2-thiol and TC-105 is 3-(phenylaminomethyl)-thiazolidine-2-thione.

These studies have brought out one further point, namely, the mechanism by which iodide is trapped in the gland. Various investigators have agreed that under thiouracil therapy the iodine accumulated in the gland due to concomitant iodide therapy was chiefly in the "inorganic" form. Studies by Salter (132), however, indicate that this so-called inorganic fraction is not inorganic altogether. It seems to consist of two sub-fractions, i.e., the free iodide,  $I_F$ , and the bound iodide,  $I_B$  (128). In brief, when iodide penetrates the cells of the thyroid, some of it is picked up by a colloidal system which forms an easily dissociable complex. Presumably this colloidal system activates the iodide and then passes it on to the protein to form diiodotyrosine. The presumption is suggested that this complex is part of an iodase enzyme system, the purpose of which is to transform iodide into the equivalent of free iodine. This problem has been studied further by VanderLaan and his associates (151), through whose courtesy the following findings are available. Their data indicate that iodine is taken up as iodide on the basis of ultrafiltration and polarographic studies; that iodide is taken up by the thyroid of hypophysectomized rats, and that this uptake is inhibited partially by thiocyanate. In their studies thiouracil appeared not to prevent the uptake of iodine but rendered the thyroid gland unable to retain iodine. They cite some data of Leblond and of Chaikoff as indicating that the percentage uptake of iodine varied inversely with the size of the dose: so that when plotted on a double logarithmic basis there was a linear relationship between dose and uptake. They interpreted these data as indicating that iodine must be present in the rat only in minute quantities and largely in the thyroid gland. It appears that

thyrotrophin is essential to hormone synthesis, which is inhibited by thiouracil. Finally, thyroid hormone secretion is unaffected by thiouracil and thyrotrophin is essential for this purpose.

#### J. IODIDE AND THE THYROID GLAND

In an earlier section three general levels of iodide intake have been categorized as (i) the natural; (ii) the "prophylactic" and (iii) the "therapeutic" (i.e., suppressive). For the moment the tremendous "fibrolytic" levels maintained in treating infectious granulomata will be dismissed. It is convenient to regard the normal thyroid gland as constantly struggling against a mild goitrogenic effect. The presence of goitrogens in common diets, as pointed out by von Suk (146) and by Astwood (11), have been discussed earlier. In dosage not too far above the "natural" level, iodide leads to an adequate formation of thyroid hormone. In consequence, the secretion of pituitary thyrotrophin declines and the thyroid tissue reverts toward the normal state. Indeed, the wide-spread use of prophylactic iodide in the control of simple goiter in the goiter belts instituted by Marine and Kimball (85) is based upon this effect. In slightly higher levels of iodide intake, corresponding to the "prophylactic" level of dosage, a stimulation of thyroxine-production occurs. This effect is doubtless related to the European phenomenon called "Jodbasedow" which has been discussed in an earlier section. In animals it may be produced easily if some degree of thyroid hyperplasia is already present. For example, evidence of hyperthyroidism was produced in goitrous dogs (86) and rabbits (157). At a still higher level, corresponding to the "therapeutic" level of iodide metabolism, the hyperplasia of the gland is suppressed much as is the enlargement of the simple pubertal goiter. Moreover, under such circumstances, the content of thyrotrophin in the pituitary is reduced just as it is in normal animals (80a). In laboratory animals the picture can be imitated by administering thyrotrophin without iodide to control animals, while the test animals receive both thyrotrophin and iodide in moderate dosage. This observation has been confirmed by numerous investigators, among whom the following are representative: Silberberg (141), Friedgood (54), and Trikojus (150). Indeed, the oxygen uptake of surviving slices of thyroid tissue removed from guinea pigs treated with a combination of thyrotrophin plus iodide, is less than after treatment with thyrotrophin alone (153). This reversal of iodide effect upon the gland at increasing levels of dosage has been described by Asher Chapman (27) and by Levine, Remington and von Kolnitz (79). Unfortunately, the successive dosage levels merge imperceptibly one into another; so that the net result is often determined by secondary conditions in the experimental plan.

These antithetical effects of iodide upon the gland have led Rawson to

present a "dual theory of iodine action". In brief, Rawson assumes that iodide can affect the thyroid parenchymal cells directly. In addition, as described in the preceding section, he assumes that elementary iodine (or its equivalent) is formed and inactivates the thyrotrophin to produce an inert iodo-protein. A similar interpretation has been advanced by Wright and Trikojus (166). Indeed, after the administration of thiouracil, both Salter, Cortell and McKay (132) and McGinty and Sharp (95) found that high doses of iodide partly inhibited the formation of goiter. An ingenious explanation of the classic action of compound solution of iodine in exophthalmic goiter has been advanced by McClendon, Foster and Cavett (89a). The thyroid and pituitary glands of twelve-week-old chickens were studied for size and for protein-bound iodine after the administration of iodine in excess and of thiouracil (0.2% in the feed). On administering thiouracil the protein-bound iodine of the thyroid was reduced by 90 per cent and that of the pituitary by 15%. On the other hand, "Lugol's solution" increased the thyroid value 133 per cent and the pituitary value 48 per cent. These authors assume that the higher protein-bound iodine in the pituitary might inhibit the secretion of thyrotrophin in exophthalmic goiter. Through the use of tracer iodide,  $I^{131}$ , Wolff and Chaikoff (163a) noted the inhibitory action of excessive amounts of iodide upon the synthesis of diiodotyrosine and of thyroxine in the thyroid gland of the normal rat. Much more diiodotyrosine was manufactured by the thyroids of rats receiving 5 or 10  $\mu\text{g}$ . than when ten to twenty times the amount was injected. Moreover, the conversion of diiodotyrosine to thyroxine was retarded when the larger quantities of iodide were injected. By working with bilaterally nephrectomized rats, Wolff and Chaikoff were able to maintain the plasma iodide concentration at 200  $\mu\text{g. \%}$  for some 40 hours. Practically none of this labeled iodide was converted to organic iodine. When the plasma concentration was as low as 15  $\mu\text{g. \%}$ , on the other hand, nearly all the labeled iodide became organically bound.

Many other theories have been advanced to explain the beneficial effect of heavy doses of iodide in exophthalmic goiter. Obviously this level of dosage is in the "therapeutic" range, described earlier. It must be confessed at the start that no final explanation is forthcoming as yet. Certain features of the phenomenon, however, are clear. The most important of these, pointed out by Salter (124), is that as the patient continues to improve and as the concentration of circulating hormone declines, the gland proceeds to manufacture active hormone at a rapid rate. This hormone is stored within the thyroid follicle, and only after the follicle is filled does the hormone proceed to "escape". Obviously, an important effect of heavy dosage of iodide is to prevent the release of freshly manufactured endocrine. Originally Salter (124) described this predominance of

"endocretion" over excretion (or secretion) as a mass law phenomenon. Moreover, Salter and Pearson (126) produced an artificial thyroid protein (Plastein) *in vitro* by altering the thermodynamic conditions of the system involved. Consequently, a large molecule was produced which could not escape through semi-permeable membranes. Subsequently, De Robertis and Nowinski (35) found that the proteolytic enzyme derived from the thyroid follicle is inactivated by treatment with elementary iodine. Whether or not this is the mechanism by which the release of hormone is prevented, it is clear the proteolysis of the thyroid colloid fails to occur during the beneficial action of iodide therapy in clinical hyperthyroidism. The same statement can be made with respect to the effect of iodide upon guinea pigs receiving thyrotrophin as studied by Friedgood (54). Some investigators have explored the possibility that the synthesis of fresh hormone in Graves' Disease is prevented by heavy doses of iodide. For example, Morton, Chaikoff and Rosenfeld (105) found that when surviving slices of thyroid tissue were exposed to a high concentration of iodide the formation of thyroxine was diminished. Such observations, however, appear not to apply to the human gland in thyrotoxicosis.

#### K. OXIDATION-REDUCTION IN THE GLAND

The oxidation-reduction properties of the thyrotrophin-thyroid system have been studied in part by De Robertis (121), but at the present time no very careful thermodynamic measurements have been reported. One must be very careful, however, in thinking of iodide, i.e., the "I" fraction of thyroid, to distinguish between freely movable ions and ions which are bound to a colloidal system. It should be remarked in this connection that Chaikoff and his associates (104,48) pointed out that slices of thyroid could accumulate iodide from an artificial medium even in the presence of goitrogenic drugs (51).

Furthermore, it has been demonstrated (162) that thiouracil actually is stored in the gland temporarily, and that this storage is decreased by the thyrotrophic hormone, and increased by potassium iodide. Astwood and Lowenstein (15) and Miller (100) have shown that on surveying the oxidation-reduction potentials of series of speculative goitrogenic agents, one can partially screen out those which will *not* be effective on the basis that they cannot reduce free iodine to iodide. This finding suggests that the effects of the goitrogenic agents is to interfere with an enzymic complex concerned with oxidation.

#### L. SOMATIC EFFECTS OF ANTITHYROID DRUGS

As pointed out earlier, thiouracil or thiocyanate in high dosage can kill acutely by inhibiting general cellular oxidation. The distinction between

possible effects on the cytochrome system and other enzymes has been reviewed. In milder dosage, the primitive cells of the bone marrow appear to be outstanding in their susceptibility; and a fatal agranulocytosis may result from thiouracil, if not from propylthiouracil. At still lower dosage, over many months, the organism sinks into profound myxedema. Salter (131) has reported the fate of rats treated with thiouracil for only 8 months. These animals gradually became torpid, sleepy and comatose; and died with thyroid glands weighing 20 times the normal value. In a previous chapter the results of Salter's associate Barnett have been reported on the suppression of fetal development, particularly in the nervous system. In Table V are given values for the "thyrenzyme" iodine found in the peripheral tissues: showing the marked decline in the concentration of this essential catalyst. Likewise, the retardation of growth in mice produced

TABLE V  
IODINE ( $\mu$ G. PER 100 G.) IN FRESH MUSCLE\*

	Fresh Muscle
<i>Normal rats (twenty)</i> . . .	$6.2 \pm 0.9$
<i>Treated with thiouracil</i>	
1.00 g. daily for 10 days . . .	5.0
1.00 g. daily for 28 days . . .	4.9
0.25 g. daily for 90 days .. .	3.2
0.25 g. daily for 105 days . . .	1.8
<i>Treated with thyroxine</i>	
0.25 mg. daily for 5 days . . .	8.2
0.25 mg. daily for 11 days .. .	20.0
0.50 mg. daily for 10 days . . .	21.0

\* From Salter, W. T. and Johnston, MacA. W. *J. Clin. Endocrinol.* **8**, 924 (1948).

by treatment after birth has been previously noted. A curious species difference, however, has been described by Mayer (87), who studied the effect of propylthiouracil on growing beagle puppies. After the age of 5 weeks these animals received large daily doses of 6-n-propyl-2-thiouracil until they were around 7 months old. Control litter mates of the same sex were maintained throughout the experiment. When the animals were sacrificed, their thyroid glands exhibited the morphological picture of marked activation, whereas the control thyroids were in the resting or storage stage. Surprisingly enough, the test puppies did not differ significantly from the control animals in general health, rate of growth, appearance, gross behaviour, relative weights of various organs, ossification of ribs and tibial epiphyses, or amount of fat stored in subcutaneous and intraabdominal depots. Only a retardation in spermatogenesis occurred,

and this disappeared after the seventh month. The uniqueness of this observation, however, is in harmony with the general finding that in most breeds of dog complete thyroidectomy (in puppies) does not lead to dwarfism. In young rats, Evans (47) found an intermediary situation: in that growth and development gradually subsided. In the development of tadpoles, however, as shown in Fig. 11 the thyrotrophin appears to be highly important.

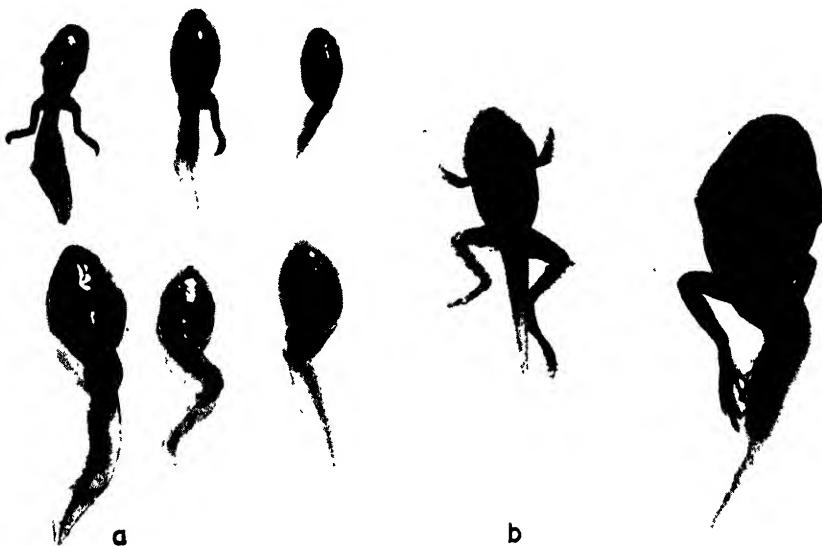


FIG. 11. a. Note the larger size, arrested development, and crooked tail base condition in the tadpoles immersed in 0.03% thiourea for 58 days since the 20-30 mm total length stage (lower animals) as compared with normal *Rana pipiens* larvae in several stages of development (top animals).

b. Recently metamorphosed *Rana pipiens* larvae. Animal on the left is a normal frog 104-days old. Animal on the right had been in 0.03% thiourea for 112 days since the 25 mm total length stage and then returned to water for 102 additional days (chronological age, 232 days). Note the marked difference in size. (From Goldsmith, Phylogeny of the Thyroid, in Thyroid Function as Disclosed by Newer Methods of Study. *Ann. N. Y. Acad. Sci.* **50**, 305, 1949.)

#### M. PERIPHERAL BLOCKING AGENTS

As pointed out above, various attempts have been made to diminish thyroid function in the organism. These have involved different approaches, i.e. direct blocking of the thyroid gland, cessation of the pituitary's thyrotrophic stimulation thereof, and antibody formation to neutralize the circulating pituitary or thyroid hormones. Theoretically there is one more possibility of antagonizing the thyroid hormone, namely, to

interfere with its operation in peripheral tissue. This approach has been studied by Woolley (164), in accordance with that investigator's general philosophy (165) that pharmaceutical agents may be produced by altering the chemical structures of vitamins or hormones in different ways. He suggested that such inhibitory structural analogs might interfere with the normal operation of hormones within the body, perhaps through competitive blocking of the enzyme systems in the peripheral tissues upon which hormones operate. Woolley, indeed, has synthesized several new ethers of *N*-acetyl-diiodotyrosine, and has found that some counteract the pharmacological effect of thyroxine. These compounds were tested on tadpoles by the method of Gaddum (55) and by the acetonitrile test of Reid Hunt (66). Some of the compounds were shown to protect tadpoles against the lethal action of thyroxine. They also antagonized the effect of the hormone in causing an increased rate of metamorphosis in these animals. The *p*-nitro-phenyl-ethyl ether was the most active, followed closely by the *p*-nitrobenzyl ether. The benzyl and butyl ethers were less effective. The nitro ethers had a weak thyroxine activity (in either tadpoles or mice) as well as an antithyroxine property. *N*-acetyl-diiodotyrosine, and *o*-methyl-*N*-acetylthyroxine were ineffective as antagonists of thyroxine. As yet, compounds of this type have not been tested in clinical material so that there is no way of knowing at the time of present writing (1949) how useful this approach may prove in human thyrotoxicosis. In a previous section further developments along these lines have been described. In particular, the work of Frieden and Winzler (52) and that of Cortell (31) indicate that more distortion of the thyroxine molecule is possible than originally appeared permissible. It must be confessed, however, that the residual activity in the distorted thyroxine-like derivatives is extremely small. As in Woolley's work (164) some of these compounds have the combined, paradoxical property both of blocking the peripheral action of thyroxine on tissues and of exhibiting weak thyroxine-like action.

Among the compounds synthesized by Niemann and tested by Freiden and Winzler in California and Ruth Cortell in the East, some contain fluorine instead of iodine. In some, the halogen is affixed to unusual carbon atoms, e.g., 4' and 6'. In others, the alanine side chain has been replaced by certain chemical radicals like the benzoyl group. A number of these compounds show weak activity like that of thyroxine, including the following: 3,5-diiodo-4-(3',5'-diiodo-4'-hydroxyphenoxy)benzoic acid; 3,5-diiodo-4(4'-hydroxyphenoxy) aniline; 3'-fluoro-3,5-diiodo-*dl*-thyronine; 3',5'-difluoro-3,5-diiodo-*dl*-thyronine; 3'-fluoro-5'-iodo-3,5-diiodo-*dl*-thyronine and 3',5'-diiodo-4-(4'-hydroxyphenoxy)3,5-diiodohippuric acid. Among seven such thyroxine analogues, Cortell found one that exhibited anti-

thythyroxine activity, namely 2'6'-diiodothyronine. This substance prevented the inhibiting effect of thyroxine and thyroglobulin on the hyperplasia of the rat's thyroid gland produced by treatment with thiouracil. Nevertheless it showed no thyroxine-like activity on thiouracil-treated rats.

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## CHAPTER VI

### Physiology of the Gonadotrophins

BY HERBERT M. EVANS AND MIRIAM E. SIMPSON

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### I. Introduction

If hypophysectomy is performed in immature animals, there is an indefinite prolongation of sexual infantilism, whereas if the operation is performed in an adult there is an atrophy of the reproductive system. The concept of the relation between the pituitary and the gonads is further strengthened by the precocious sexual maturity which may be produced by the implantation of anterior hypophyseal tissue into immature rats or mice. One can produce not only the precocious establishment of ovulation but an accentuation of normal processes, *i.e.*, superovulation.

Effort was early made to separate the hormone or hormones in anterior pituitary tissue which thus presided over the development and function of the reproductive system. It is unfortunately true that most crude extracts as well as implants of the anterior pituitary do not set up orderly or normal processes in youthful ovaries. Disorder or abnormality is evidenced by a production of lutein tissue in follicles which have not ovulated, *i.e.*, discharged their eggs—so that the corpora lutea thus produced contain ova. The discovery by Fluhmann (87) and by Zondek (279) that the blood stream and urine of women in the menopause contain a gonadotrophic hormone producing chiefly, if not exclusively, growth of the ovarian follicles led naturally to the concept that the stimulus to follicular growth and to luteinization might come from different gonadotrophic hormones.

Early efforts at the fractionation of gonadotrophic extracts from the pituitary appeared to have effected an approximate separation of the follicle stimulating and luteinizing properties and the fractions were consequently said to consist predominantly of the follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively. Although the female had been used as the original test object, it was early recognized that these

substances affected the male. The pioneer exploratory work seemed to show that the follicle stimulating fraction primarily supported the germ cell producing (spermatogenic) activities of the testis, and the luteinizing fraction the hormone-producing (hormonogenic) activities of the testis.

In the meantime, attention had been given to the highly characteristic type of degeneration of the interstitial tissue in the gonads of both sexes after hypophysectomy. This degeneration consisted not only in general shrinkage of the interstitial cells but in an agmination of the chromatin of the nuclei of these cells, so characteristic that it might be recognized even with low powers of the microscope. In the ovary, they were designated "deficiency cells" (Selye) (Fig. 1). The Leydig cells of males were seen to undergo the same change (Simpson). Pituitary gonadotrophic extracts could be produced which would restore the deficiency cells without pronounced effects on the ovarian follicles (Fig. 2). The gonadotrophic hormone involved was hence designated the interstitial cell stimulating hormone (ICSH). It would appear that such a designation is better than luteinizing hormone (LH) for the production of lutein tissue occurs only in the female. Conversely, extracts could be produced which would cause marked growth of the ovarian follicles without effect on the interstitial tissue (Fig. 3).

Although, as has just been stated, extracts of anterior pituitary tissue can be made which unfold these two separate gonadotrophic effects, it is by no means certain that the pituitary secretes two distinct gonadotrophic hormones—substances identical with or resembling the two hormonal substances which the biochemist has isolated from pituitary tissue—or that they are both necessary in the two sexes for normal functioning of the reproductive system. It is particularly to be noted that no *pituitary*<sup>1</sup> hormone whose predominant effects are interstitial cell stimulation or luteinization has been found in the blood stream or urine.

## II. Pituitary Gonadotrophins (FSH and ICSH)

### A. EFFECTS IN THE FEMALE

Greep, van Dyke, and Chow (109) reported the preparation of FSH in "nearly pure" form. Fraenkel-Conrat, Simpson, and Evans (96), and others in the Berkeley laboratory were unable to completely purify the hormone completely, although it was effective in as low or lower doses than the preparation of the Greep group (3 to 5 microgram doses). Multiples of 10 to 40 times the minimum dose, especially when injected intraperitone-

<sup>1</sup> As will be noted when the topic is discussed, such a hormone is, strangely enough, fabricated by the chorion of the human embryo. The substance, human chorionic gonadotrophin, is *not* a pituitary product.

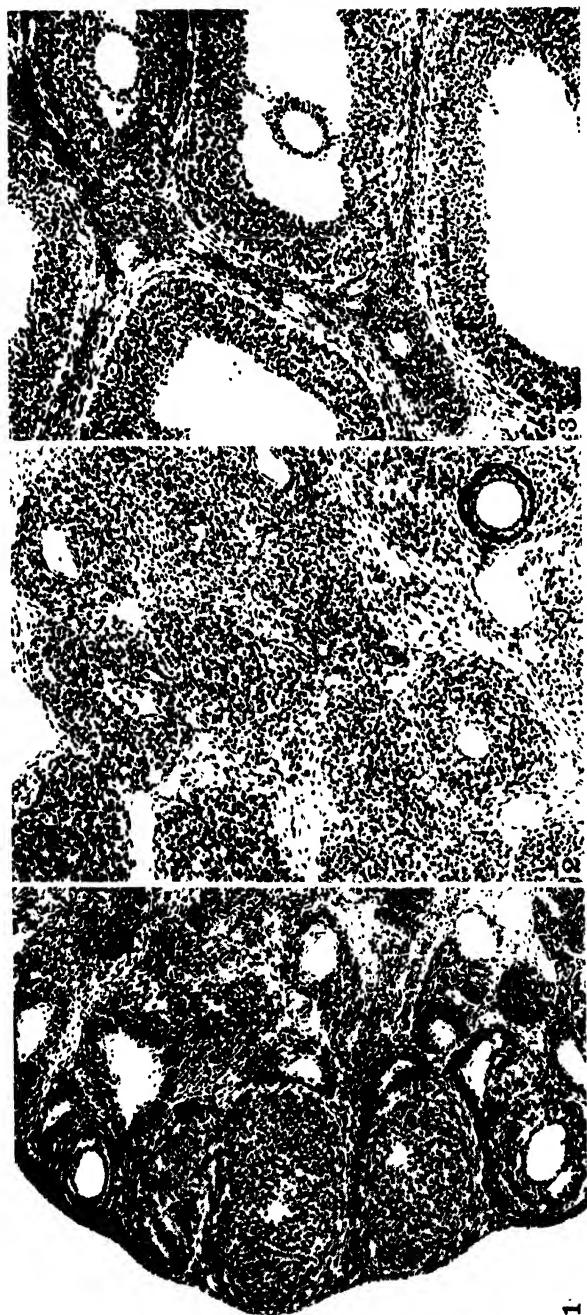


FIG. 3

FIG. 1.—Ovary of uninjected hypophysectomized rat, 27 days of age at operation, 11 days postoperative, the ovarian follicles never progress beyond the size of the two largest shown (beginning antrum formation); the characteristic black deficiency cells are clearly shown.

FIG. 2.—Ovary of hypophysectomized rat of the same post-operative age, injected with pituitary ICSH for 3 days previous to autopsy; the nuclei of the interstitial cells are now normal and each cell possesses an appreciable amount of cytoplasm

FIG. 3.—Ovary of hypophysectomized rat similarly treated with pituitary FSH, the ovarian follicles (five of which are shown) are greatly enlarged though they never constitute fully ripe Graafian follicles. The interstitial cells (between the follicles) are still deficient.

(After Li, Simpson, and Evans, 1935, and Evans, Simpson, and Penchatz, *Cold Spring Harbor Symposia Quant Biol.* **5**, 229, 1937.)

ally, showed the presence of biologically active contaminants, notably ICSH. Li, Simpson, and Evans (176) have recently obtained FSH in electrophoretically homogeneous state.

The principal biological property of this hormone in the female is the stimulation of development of multiple follicles. Greep, van Dyke and Chow (111) reported that though follicles increased in size on injection of pure FSH, they were unable to reach full size or to secrete estrin (as measured by the response of the uterus and vagina). Addition of LH immediately resulted in estrin production. This appears to be true of all but the purest FSH, estrin effects not being shown until doses are large enough for the effects of ICSH contamination to begin to display themselves.

Since the preparation of ICSH in pure form by Shedlovsky *et al.* (238), Li, Simpson, and Evans (174,175) and Greep, van Dyke, and Chow (110), it has been possible to determine the biological properties of this substance also with reasonable accuracy. In the female rat, ICSH repairs the deficient interstitial tissue resulting from hypophysectomy. The uterus and vagina show no evidence of stimulation. When given to the normal rat, increased amounts of lutein tissue occur and the corpora lutea are functional. ICSH given simultaneously with FSH causes augmented ovarian responses. In the hypophysectomized rat, the augmentation occurring at low doses shows first in enlargement of follicular size. The augmentation may occur at doses of ICSH so low that the interstitial cells are still atrophied with pyknotic nuclei. At higher doses, follicles ripen and luteinization occurs. Combined in the proper proportion (ca. 10 to 1) FSH and ICSH result in ovulation. This synergism is evident when ICSH is administered in combination with FSH subcutaneously, or upon injection of the two substances at separate sites subcutaneously. Non-specific augmentation of the follicle stimulating effects may be obtained by the addition of biologically inert substances, such as copper salts. The components must be injected together to obtain such non-specific synergism.

Injected intraperitoneally into normal animals, ICSH antagonizes the gonadotrophic action of the subcutaneous dosage with all other known gonadotrophins. This antagonistic effect of intraperitoneal ICSH does not seem to occur in hypophysectomized animals (Fraenkel-Conrat *et al.*, 94).

#### B. EFFECTS IN THE MALE

Earlier reports that FSH stimulates spermatogenesis in the hypophysectomized male rat may be questioned, due to the undoubtedly contamination of the earlier preparations with small amounts of ICSH. There was subsequently shown a surprising sensitivity of the testicular tubules to a

dosage with pure ICSH so low as to be only a fourth of that necessary to repair the interstitial cells, spermatozoa appearing but the male accessories remaining atrophic for the 15 days of the experiment (Simpson, Li and Evans (242)). A preparation from anterior hypophyseal tissue giving only FSH effects in the female has now been prepared as a pure protein, judged by physico-chemical characteristics. It has definitely led to the appearance of spermatozoa in the testis of hypophysectomized rats, while the male accessories are still atrophic.

The properties of ICSH in the male as described by Shedlovsky *et al.* (238), and Greep, van Dyke, and Chow (111), include stimulation of the interstitial cells of the hypophysectomized male rat, increase in weight of the ventral prostate (at a dose of 1 microgram hormone nitrogen or 6.7 micrograms of hormone) and an increase in the weight of the testis (at twice that dose). Fraenkel-Conrat, Li, Simpson, and Evans (95), and Simpson, Li, and Evans (241), reported the following effects of ICSH: in the hypophysectomized male rat, it repairs the interstitial tissue and enables it to secrete, so that the accessory organs of reproduction are increased in size. It also allows spermatogenesis to proceed at the normal rate in male rats hypophysectomized before sexual maturity.

### C. BIOASSAY OF PITUITARY GONADOTROPHINS

Emerging from these biological properties, the test methods of choice for ICSH have been: 1) repair of the interstitial tissue of the ovary (the dose required for repair of Leydig cells in the male is almost identical) and, 2) the weight increase in the ventral prostate of the hypophysectomized immature male rat. The various methods which have been used are shown in Table I. It will be possible to see in this table the relative sensitivity of these methods of standardization and the value of the unit in terms of pure substance.

Two methods of assay of FSH have proved most useful: 1) Determination of the minimum dose effective in reestablishing follicular growth in the ovary of hypophysectomized rats—*i.e.*, microscopically detectable growth. 2) Augmentation of ovarian weights of immature normal rats by injecting it in combination with human chorionic gonadotrophic hormone (HCG). Augmentation of ovarian weights by 100% occurs at approximately the minimum dose detectable in hypophysectomized females, the two units being therefore approximately the same (see Table II).

The augmentation in ovarian weights caused by combining FSH either with ICSH or HCG (Tables III and IV) illustrates the phenomenon of *synergism* which is evidenced by a combination of the two pituitary gonadotrophins (FSH and ICSH) as well as by a similar combination of the principles found in menopause and in pregnancy urine; the last mentioned substance, human chorionic gonadotrophin, may be used as well as

pituitary ICSH to augment effects from pituitary FSH. Spectacular is the sudden increase in the weight of the uterus which is now fluid filled and

TABLE I  
COMPARISON OF METHODS FOR STANDARDIZATION OF PITUITARY ICSH

Test	Unit Total dose in mg.*
Immature female rats (26 day) hypophysectomized Repair of deficient interstitial cells (3 days, I.P.)	0.035
Augmentation of FSH (Injected, 7-10 days p.o. for 3 days subc.) ..	0.001
Immature male rats (23 day) hypophysectomized (Injected, 2 days p.o., for 4 days, subc.) Ventral prostate, 100% increase..... Testis weight, 100% increase .. .	0.050-0.100 0.50
Immature pigeon (33 days) (Injected for 4 days intramusc.) Testis weight, 100% increase .. . . .	0.030
Chick (1 day) (Injected for 5 days, subc.) Testis weight, 100% increase .. . . Testis, histological .. . .	5.0 0.1

\* Of the pure substance (Li, Simpson, and Evans, 175) as prepared from sheep whole pituitary tissue.

TABLE II  
COMPARISON OF METHODS FOR STANDARDIZATION OF PITUITARY FSH

Test	Unit Total dose in mg.*
Immature female rats (26 days), hypophysectomized Reestablishment of follicular growth (histological) (Injection 7-10 day p.o., for 3 days, subc.)	0.003
Normal immature female rats (24-25 days) Augmentation combined with standard dose HCG (for 3 days, subc.)	0.003

\* Of the pure substance as prepared from sheep whole pituitary tissue (Li, Simpson, and Evans, 176).

in the estrous condition due to the secretion of the female sex hormone by the stimulated ovary.

TABLE III

AUGMENTATION OF FSH BY ICSH IN IMMATURE HYPOPHYSECTOMIZED RATS  
72 HOUR TEST: SUBCUTANEOUS ADMINISTRATION

Hormones	Ovaries			Uterus	
	Weight	Description		Weight	Description
	mg.	mg.		mg.	
FSH $\frac{1}{2}$ RU)	0.001	8	No F devel. IT deficient	21	Small
ICSH $\frac{1}{2}$ RU)	0.001	9	No F devel. IT deficient	22	Small
FSH + ICSH	0.001 0.001	35	s,m,ml F. IT deficient	122	Estrous
Control		8	sF IT deficient	24	Small

FSH 1 RU = 0.003 mg. subc.; 10 RU (0.03 mg.) causes estrous uterus.

ICSH 1 RU = 0.005 mg IP (MED subc. = 0.025 mg.)

Modified after Fraenkel-Conrat, Li, Simpson, and Evans.

Key to abbreviations used in this and subsequent tables:

F, follicles or follicular	def, deficient
CL, corpora lutea	dev, developed or development
IT, interstitial tissue	incr, increase
RU, rat units	s, small
est, estrous	m, medium
lut, luteinization or luteinized	l, large

TABLE IV

SYNERGISM BETWEEN FOLLICLE STIMULATING HORMONE (FSH) AND  
HUMAN CHORIONIC GONADOTROPHIN (HCG) IN IMMATURE  
HYPOPHYSECTOMIZED RATS

96 Hour Test—Subcutaneous Administration

Treatment	Ovaries			Uterus	
	Total dose	Weight	Description	Weight	Description
	mg.	mg.		mg.	
FSH . . . . .	0.005	10	Few S,m F IT deficient	24	Small
HCG . . . . .	0.050	12	No f dev IT repaired	22	Small
Combination . . .	50	m, ml F	IT repaired	120	Estrous
Control . . . . .	8	sF	IT deficient	24	Small

Modified after Evans, Fraenkel-Conrat and Simpson.

**D. RELATION OF THE SEX HORMONES TO THE PITUITARY GONADOTROPHINS****1. Female Sex Hormone**

*a. Indirect Effect of Estrogen.* It is known that the pituitary causes estrogen secretion by the ovary. Estrogenic substances in turn have an effect on the outpour of gonadotrophic substances by the pituitary. Estrogen administered to immature rats leads to corpus luteum formation (Hohlweg, 142) which was interpreted as due to outpour of LH from the pituitary as this effect of estrogen does not occur in the absence of the pituitary. The outpour of LH from the pituitary does not persist, according to Hellbaum and Greep (128). The pituitaries of ovariectomized rats which had been treated with estrin over a 30-45 day period did not stimulate corpus luteum formation but only follicular development. Fevold, Hisaw, and Greep (84) have even described a diminution in estrogen effects within 8 days. As will be seen later, this relationship has been used by Hisaw (137), Fevold (83) and others in explanation of the estrous cycle. The further assumption must be made that estrogens check further elaboration and outflow of FSH. Where unchecked, as in the castrate, the pituitary not only contains great amounts of follicle stimulating hormone, but is also pouring it out in great quantities as shown by parabiotic twins. The hypertrophy of the ovary and eventual tumorous transformation observed in rats whose ovaries have been transplanted to the spleen is interpreted as due to continued outpour of gonadotrophins from the pituitary which has been released from estrogen control due to blood drainage from spleen into liver followed by estrone inactivation in the liver (Heller, Jungck, Nelson, and Winter (132), Mark and Biskind (183)).

The corpora lutea induced by estrogens in an animal possessing a pituitary are functional as can be shown by the maintenance of diestrus, mucification of vaginal epithelium, proliferation of the endometrium and the ability of this endometrium to nidate a foreign object, a decidioma being formed. An additional mechanism is, however, involved. It can be shown that even though morphologically normal appearing corpora lutea are formed that they are not functional unless a further pituitary principle is present (Robson (214,215,216), Astwood (13), Evans *et al.* (75)). If hypophysectomy is performed immediately after ovulation and corpus luteum formation, the uteri of such animals will not produce decidiomata. Injection of crude pituitary extracts will make the corpora lutea functional. Evans, Simpson, Lyons, and Turpeinen (76) and Nelson (198) found that the pituitary principle which was effective was the lactogenic hormone. The former group used electrophoretically homogeneous lactogenic hormone; the latter worker found the capacity to maintain pseudo-pregnancy paralleled other measures of lactogenic potency. Astwood (13) perhaps influenced by the efficacy of placental extracts (Astwood and Greep (14)),

decided that possibly a hormone separate from other known pituitary principles established the functional state of the lutein cells.

b. *Direct Effect of Estrogen on the Ovaries.* The effect of estrogen in the production of functional corpora appears to be indirect, and due to the stimulation of the pituitary to pour out luteinizing hormone and lactogenic hormone (luteotrophic substance). A direct effect on the ovary has also been demonstrated. Williams (276) showed that estrogen could induce considerable follicular enlargement in hypophysectomized rats. Pencharz (203) showed that this effect on the hypophysectomized rat's ovary enhanced the ovarian response to equine gonadotrophin. Simpson *et al.* (240) showed synergism between estrin and pituitary gonadotrophins in the hypophysectomized rat.

c. *Hormonal Control of the Estrous Cycle.* The reciprocal action of estrin on the pituitary has been used to explain the rhythmicity of ovarian function. The commonly held concept is as follows: FSH stimulates follicular growth, synergized by small amounts of ICSH, to produce follicular ripening; the estrin produced decreases the FSH output from the pituitary and releases larger quantities of ICSH, (LH) which luteinize the ovaries; now the outpouring of lactogenic hormone (luteotrophic hormone), under the same stimulus, renders the corpora lutea functional. The possibility exists that lactogenic hormone may also be necessary for ovulation (Everett (78,79)). It is further possible that FSH and LH are not adequate for normal luteinization—luteinization of both theca and granulosa. As estrogen production decreases after ovulation the pituitary again secretes FSH in larger amounts. Progesterone produced by active corpora lutea may also inhibit the secretion of pituitary ICSH (LH).

## 2. *Male Sex Hormone*

a. *Direct Action.* In 1934 Walsh, Cuyler, and McCullagh (269) found that the male sex hormone injected into hypophysectomized rats maintained the testicular tubules. This was amply confirmed by other workers, and the observation was extended to primates by Smith (247). Further, Smith (252) and Dvoskin (57,58) showed that pellets of testosterone could stimulate the testis locally without a systemic effect. These observations were difficult to understand in view of the concept which had become established that pituitary gonadotrophins controlled the gonads. Meanwhile some observers, Smith and Leonard (254,255) and Evans *et al.* (70), had found that human chorionic gonadotrophin was effective in maintaining spermatogenesis in the hypophysectomized male rat. As this substance acted in the female only to repair interstitial tissue, showing no follicle stimulating potency which was the portion of the gonadotropic complex assumed to be gametokinetic in the male, the action of chorionic

gonadotrophin in the male was not understood. These observations remained discordant until the interstitial cell stimulating hormone was isolated and was shown to be gametokinetic in low doses in the hypophysectomized male. ICSH even allowed the testicular tubules of the rat hypophysectomized at 40 days of age, and containing germ cells only differentiated as far as spermatids, to progress in their differentiation as rapidly as normal with production of spermatozoa by 55 days of age. (Table V) The conclusion was reached that it and chorionic gonadotrophin must act directly on the testes through the male sex hormone produced by the stimulated Leydig cells. But attention must be called to the fact

TABLE V

EFFECTS OF PURE ICSH ON THE REPRODUCTIVE SYSTEM OF HYPOPHYSECTOMIZED 40-DAY MALE RATS

15 Day Maintenancy Test, IP Injection

Type of rat	Daily dose	No. of rats	No. with spermatozoa	Testes	Seminal vesicles	Prostate (Total)
Hyph.	mg.			mg.	mg.	mg.
	1.000	5	3	1118	74	193
	0.250	5	2	1031	56	139
	0.050	5	5	1427	65	151
	0.010	8	6	1065	32	92
	0.0025	5	1*	743	19	59
Normal	0.0	242	0	453	18	48
	0.0	246 (40-43 days)	0*	1345	42	129
	0.0	15 (55 days)	14	2355	255	305

\* Spermatids present.

Modified after Simpson, Li and Evans (242)

that pituitary ICSH is effective in maintaining the tubules at doses which produced little or no effects on the accessory organs of the male. At higher doses, the accessory organs were stimulated.

To assume from these experiments that the seminiferous tubules need only one pituitary gonadotrophin, ICSH, and that ICSH acts as a complete gonadotrophin in this sex, controlling both tubules and interstitial cells would be premature. Though these three agents, testosterone, human chorionic gonadotrophin and the interstitial cell stimulating hormone all maintain spermatogenesis, they are much less effective in repair of this function after atrophy has overtaken the testes suggesting another factor is necessary. Furthermore, even in maintenance studies the testis itself, and

its tubules, are never quite as large as normal, again indicating that some other factor is needed. It should also be remembered in this connection that the pituitary of the male contains a high content of hormone which is predominantly follicle stimulating.

As discussed earlier, pure FSH is able to stimulate tubules and spermatozoan formation at doses barely minimal for Leydig cell repair, where the interstitial cells are more like connective tissue (not epithelioid), and seminal vesicles and prostate are as completely atrophic as in controls.

One possible implication of these apparently conflicting results is that the synergic combination of FSH and ICSH is actually needed for complete normality in the male.

*b. Indirect Action.* The observation that testosterone inhibits or injures the testis in the normal animal was at first difficult to harmonize with stimulation of testes noted in the absence of the pituitary. The double action in the normal animal, one indirect through the pituitary, and the other direct, (as shown by intratesticular pellets) was adequately demonstrated by Jensen, *Anat. Record Suppl.*, 100, 48 (1948). Small doses of testosterone were shown to be inhibitory, acting through the pituitary to reduce its gonadotrophic content, as shown by implant studies to assay their potency. Large doses, though decreasing the pituitary content of gonadotrophin, had also a direct effect on the testis, as observed on the injection of large doses or implantation of pellets of testosterone into hypophysectomized rats.

#### E. OTHER FACTORS AFFECTING PITUITARY GONADOTROPHIC SECRETION

Beside the effect of the sex hormones on pituitary gonadotrophic production, as seen in castration and cryptorchidism, after the menopause, or in primary hypoplasia of the gonads, or after injection of estrone or testosterone, other conditions are known to affect the pituitary section. *Tumors* of the pituitary, such as eosinophile adenomata, are associated with decreased secretion. *Brain-pituitary* relationships are beyond the scope of this chapter. Markee and others (184,185) have shown a stimulation of the pituitary output of gonadotrophin by direct injection into the pituitary of epinephrin and related substances. Both the secretion of gonadotrophins and the response to them vary with *age*. One illustration only will be given, the age relation to responsiveness to gonadotrophins. Very young rats, before 18 days, do not respond with follicular development to HCG or equine gonadotrophin, though the interstitial tissue can be rendered hyperplastic at birth or before; the ovaries of new-born colts are larger than the maternal ovaries and are composed of masses of hypertrophic interstitial cells. A possible interpretation of the changed response in the rat at 18-21 days of age is that it marks beginning secretion of FSH by the pituitary. (HCG is synergic with FSH and equine gonadotrophic

hormone is known to be a more potent agent in the presence than in the absence of the pituitary.)

*Seasonal influences* in gonadotrophic production and outpour have been shown to be of importance, especially in seasonally breeding animals. The gonadotrophic hormone content of the pituitary of the ground squirrel during the period of aspermia is decreased (Wells, 274). *Light* is one of the important seasonal factors influencing pituitary gonadotrophin. Light stimulation of reproductive activity in birds has been known for some time, a stimulus not acting in the absence of the optic nerve. A similar stimulus by light to reproductive activity in mammals has been shown by demonstration of changes in ovaries and in reproductive performance. The ferret, for example, exposed to an increased number of hours of light shows increased reproductive activity (Bissonnette, 18); this effect is not shown after section of the optic nerve (Hill and Parkes, 135).<sup>2</sup>

*Inanition* and *vitamin deficiencies*, especially in the B complex, lead to definite atrophic changes in the gonads, which have been interpreted as effective through the pituitary, in some cases on the basis of the pituitary potency by implantation methods.

### III. Human Urinary Gonadotrophins

#### A. HUMAN CHORIONIC GONADOTROPHIN (HCG)

##### 1. *Properties*

The first known naturally occurring gonadotrophin was that found in the blood and urine of pregnant women—human chorionic gonadotrophin—originally designated “Prolan” by Aschheim and Zondek, its discoverers. Its presence was detected by its ability to produce precocious maturity (estrus, follicular development, ovulation and luteinization) in immature rodents, rats and mice, within a period of 96 hours. The hormone was not at first distinguished from pituitary hormone. In fact the terminology of its discoverers reflects this conception. Several types of work led to the realization that HCG is not identical with any pituitary hormone. The pituitary of pregnant animals was not found to contain an increased amount of gonadotrophin, as might be expected if increased amounts of gonadotrophin were being secreted in pregnancy. Other body fluids, *e.g.*, blood and urine of castrated human beings, were found to have physiological effects different from the HCG; they were found to be predominantly follicle stimulating, more like the physiologic potency of the pituitaries in such cases. Human chorionic gonadotrophin was furthermore found to

<sup>2</sup> On the other hand, Yeates, N. T. M. (*Nature* 168, 429 (1947), has shown that in ewes “a change from increasing length of day to decreasing length of day induces reproductive activity”.

be more limited in the ovarian weights which it could induce in the acute test (72-96 hour) than were pituitary preparations (see Fig. 4). The number of follicles which develop as a result of stimulation by human chorionic gonadotrophin corresponds quite closely to the number occurring in normal ovulation in this species—*i.e.*, a “crop” of follicles is produced rather than the more numerous follicles seen after high dosage with pituitary extracts. It was then found that chorionic gonadotrophin was really an incomplete gonadotrophic agent in the sense that it stimulated no follicular development in hypophysectomized rats, having as its only effect repair of the interstitial cells.

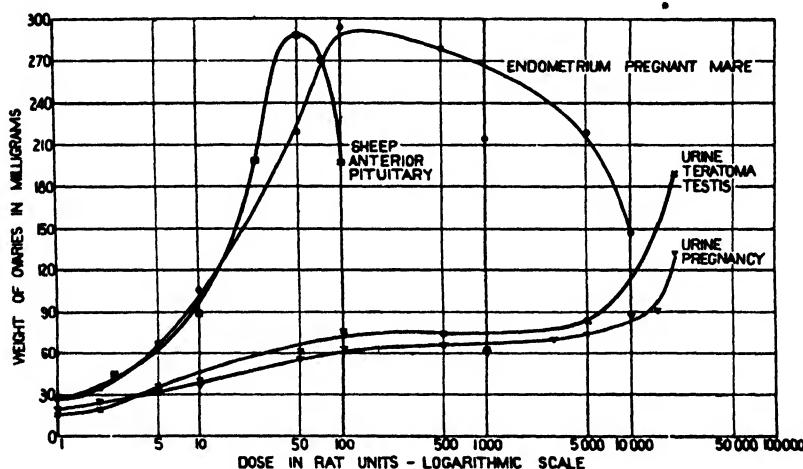


FIG. 4.—Curves showing the ovarian weight responses to increasing dosage in rat units with three types of gonadotrophic hormones (sheep pituitary, endometrium of pregnant mare, and human urinary chorionic hormone) as found in testicular neoplasm and normal pregnancy. (After Evans, 66.)

It was soon found that pituitary preparations were synergic with HCG, increasing the number of follicles developing and enlarging the ovarian weights resulting from HCG injection. This discrepancy led to a long series of studies to determine which pituitary principle was synergic with it. The pituitary principle is now recognized as the follicle stimulating fraction. The recognition of the identity of this “activating” principle in the pituitary was obscured, previous to the work of Leonard (167), by the fact that FSH exerts this effect in enlarging the ovaries, resulting from injections of HCG, at doses without effect when injected alone in the immature normal rat.

The Aschheim-Zondek test for pregnancy, in which precocious sexual maturity is induced in normal immature rodents, is based on the coopera-

tion of a pituitary secretion with the chorionic gonadotrophin present in pregnancy urine—*i.e.*, it is based on the synergism between pituitary FSH and HCG.

On administering HCG to hypophysectomized male rats, maintenance of the seminiferous epithelium and normal or exaggerated function of the Leydig tissue occurs. Such males are able to sire litters. This experience may be used to support the concept that only a single gonadotrophic hormone, ICSH, is required by the male.

The experimental demonstration that HCG is a stimulus to the Leydig tissue and hence male accessories has led to its clinical use in cases of undescended testicle in adolescent or post-adolescent boys. In a proportion of such cases this therapy is dramatically successful.

Though HCG closely resembles pituitary ICSH, there are a number of distinguishing features. HCG produces a gonadotrophic reaction in the normal immature rat, apparently acting as a complete gonadotrophin, though in reality it cooperates or synergizes with a pituitary product in creating this effect. Inasmuch as ICSH has no gonadotrophic reaction in the normal immature animal, though it is synergic with pituitary FSH just as efficiently as is HCG, it is assumed that HCG actually stimulates the pituitary to produce FSH, whereas ICSH is unable to do this, or at least is a less potent stimulus to the pituitary.

Another way in which the two substances differ is that the route of injection affects the physiological action of ICSH much more radically than does the route employed for the administration of HCG. ICSH (or some closely adherent agent, not separated from homogeneous preparations of ICSH) when injected intraperitoneally antagonizes the gonadotrophic potency of other preparations simultaneously injected subcutaneously. (It usually augments the gonadotrophic potency of these same substances when both are injected subcutaneously.) HCG injected intraperitoneally is not antagonistic to simultaneously injected gonadotrophins. This antagonism of ICSH, when injected intraperitoneally, to gonadotrophic agents simultaneously injected subcutaneously, occurs in normal immature female rats but not in hypophysectomized rats and is therefore open to the interpretation that this represents an effect through the pituitary. The potency of HCG as an interstitial cell stimulating agent is greater than that of ICSH in the sense that the interstitial cells stimulated by it (at similar multiples of the MED) are larger, more epithelial, and stain more intensely with eosinophilic dyes. The cells stimulated by HCG are also evidently producing more male hormone as judged by the greater response of the seminal vesicles and prostate. HCG and ICSH differ also in the responses incited in other species. The immature bird testes (chick, squab) increase markedly in size on injection of pituitary ICSH but are very insensitive to HCG.

## 2. *Origin of Human Chorionic Gonadotrophins*

When it was learned that the hormone in the blood and urine of pregnant women did not have the biological and chemical properties of pituitary gonadotrophin, attention was directed to the presence of large quantities of this hormone in the placenta (Hirose (136), Murata and Adache (196) and Aschheim (4)). The conviction that the hormone originates there is reflected in the current terminology for the hormone, "human chorionic gonadotrophin." That the growth curve of activity of the cytotrophoblast is in rough accord with the curves of chorionic gonadotrophin in the blood and urine, and that the highest level occurs in the placenta at the time of the highest titer in the blood and urine (2 months from onset of pregnancy) are taken as substantiating the concept of its origin in the placenta (Zondek (278), Browne and Venning (21), Smith and Smith (245), Evans, Kohls, and Wonder (72), Bickenbach (16,17), Smith and Smith (244)).

The eventual decline in the production and excretion of chorionic gonadotrophin as pregnancy progresses is said to be correlated with the decline and disappearance of the Langhans cells. This is substantiated by the same relationship in other primates where the early disappearance of the hormone correlates with early disappearance of the Langhans cells as a distinct layer. In the chimpanzee the hormone disappears from the blood between 100 and 160 days (Elder and Bruhn (59)); in the rhesus monkey it disappears 30 days after ovulation (Delfs (56)).

The first experimental evidence showing that the placenta contained the hormone was obtained by implanting bits of placenta or by injection of placental extracts (*e.g.*, Collip, 42) in rats or mice. The first evidence that the placenta produced, rather than stored the hormone was obtained by Kido (154) who made implants of placentas into the anterior ocular chamber of rabbits; the implants grew and evidence of their secretion was furnished by the fact that they characteristically influenced the ovaries.

The continuance of elevated hormone titers in the blood and urine when hydatidiform mole persists or chorioepithelioma occurs, and the presence of high titers in the male with testicular tumors having structural characteristics similar to those of chorionic tissue, also supported the chorionic origin of the hormone (see section on tumors.) Finally, the highest known tissue content in this hormone occurs in some of the purely choriomatous hepatic metastases of a primary testicular neoplasm.

Histochemical studies (Wislocki and Bennett (277)) are convincing that the cytotrophoblast is a secretory tissue.

Evidence from tissue culture is in agreement with other evidence in showing that placental chorioepithelioma, in particular the Langhans layer, produces the hormone. (Gey, Seegar, and Hellman, 105; Seegar-Jones, Gey and Gey, 232; and Stewart, Sano, and Montgomery, 258.)

### 3. Purification of Human Chorionic Gonadotrophin

Extensive and successful efforts to purify human chorionic gonadotrophin have been conducted by Doisy and especially by Gurin and collaborators (113,114,115). Preparations containing 6000–8000 IU/mg. were secured. Claesson, Höglberg, Rosenberg, and Westman (32) have recently described its crystallization. Their preparation is electrophoretically homogenous and crystalline. The unit of their preparation is 10,000 IU/mg., which is about the same as that reported by Gurin. (Claesson feels that the preparation made by Gurin and possibly also that of Doisy was substantially pure.) Study of the biological properties of the pure material have confirmed those previously known. It had been known from animal experimentation that the hormone was a stimulant to the pituitary and among the striking achievements of Claesson *et al.* from the intravenous administration of the pure hormone in amenorrheic women was the development of one or more ovarian follicles which we are probably entitled to look upon as resulting from an outpouring of pituitary FSH thus provoked.

### 4. Biological Tests for Pregnancy

The first biological test for pregnancy (Aschheim and Zondek, 7,8,9) based on the occurrence of gonadotrophic hormone in the blood and urine of pregnant women depended on the formation of corpora lutea or "blood points" in the ovaries of mice or rats within 96 hours after injection of the urine. Ovulation in the mature mouse within 18–24 hours after a single injection of urine has been proposed by Burdick (24) as a test for pregnancy. Corpus luteum formation in the immature or mature isolated rabbit within 24 hours after a single injection of pregnancy urine was introduced by Friedman (102) as a quick pregnancy test. It is the test commonly employed. Gross inspection of the ovary shows marked hemorrhage into one or more follicles.

Vaginal estrus in the immature rat, occurring 72–96 hours after onset of injection of urine, has been used as a pregnancy test (Aschheim (6)). Uterine hypertrophy in the rat has also been used (Heller, Lauson, and Sevringshaus, 129). Barlow and Sprague (15) find the following order of reliability of these tests in the rat, listed in order of decreasing reliability: vaginal estrus, uterine hypertrophy, blood points or corpora lutea, ovarian hypertrophy. Uterine hypertrophy in the mouse is the method of choice by many workers as it is in gonadotrophic assays of non-pregnancy urines. The response of the accessory organs of the male to chorionic gonadotrophin has also been used to some extent as a pregnancy test.

An international unit was adopted in 1938 (see League of Nations, Health Organisation Bulletin 1939 (162)), being defined as the gonado-

trophic activity of 0.1 mg. of a standard preparation of chorionic gonadotrophin. By its use it is possible to compare the sensitivity of these different methods.

Recently the stress has been on obtaining quicker tests than those just listed. There has been considerable interest in the utilization of the early hyperemia of the rat ovary which follows a single injection of pregnancy urine. (See Frank and Berman (100); Salmon, Geist, Frank, Poole, and Salmon (230); Salmon, Geist, Salmon, and Frank (229); Ramsey, Falkenstein, and Sujkowski (207); Farris (80,81); Aschheim and Varangot (11); Bunde (23); Zondek and Sulman (283); Riley, Smith, and Browne (210).) The ovaries have been examined for hyperemia after 2 to 6 hours, the consensus being that the longer interval is better. Zondek and Sulman (284) report in regard to sensitivity of the test in relation to time that the lower the unitage the longer the period needed for the response; 4 hours being required to detect 20 IU and 11-24 hours for 2 IU.

Three types of pregnancy tests have been devised using amphibia. The earliest depended on ovulation in frogs or toads (Shapiro and Zwarenstein (234,235,236,237), Crew (47), Weisman *et al.* (271,272,273), Foote and Seegar-Jones (92)). Ovulation in different species occurs from 6 to 18 hours after injection. Weisman and Coates (270), cite 50 RU as a stimulating dose. The second type of test, introduced by Galli-Mainini (104), depends on the discharge of spermatozoa from the testis and their detection in the cloaca. The optimal time recommended by them for tapping the cloacal contents is 3 hours. Robbins *et al.* (212,213), found the male South African clawed frog (*Xenopus laevis*) and the American frog, *Rana pipiens*, satisfactory. They report that the male African frog is 10 times as sensitive as the female rat. A high degree of reliability is claimed for these amphibian tests employing either male or female.

Guterman's test for pregnancy is based on a colorimetric determination of pregnanediol excretion; 1 mg. pregnanediol per 100 cc. of urine is considered diagnostic of pregnancy (Guterman, 116,117; McCormack, 181). The test requires 3 hours. Guterman reports 92% accuracy and finds the test positive any time after the first missed period. Morrow and Benua (194) find 25% error possible in both the presence and absence of pregnancy. Seegar-Jones, Delfs, and Stran (233) report that the titer of pregnanediol is highest in the last trimester (when the corpus luteum is no longer functional and the placenta must be furnishing the hormone).

##### 5. Time of Appearance of Human Chorionic Gonadotrophin in the Urine of Pregnant Women

The time of appearance of increased gonadotrophin titers in the blood and urine after onset of pregnancy has not been determined accurately in

many cases. We may also reckon with some possible fluctuation in the precise time of ovulation in relation to the menstrual period, itself varying. Aschheim and Zondek (7) originally reported that gonadotrophin was detectable in the urine within two weeks after the first missed menses. Since then the rise has been reported the week before, by the time of, or the week after the expected menses. The unsatisfactory state of our knowledge is reflected in Table VI. (Aschheim (5), Frank (98), Browne and Venning (22), Smith and Smith (243).) One of the most convincing accounts in the literature of the sudden increase in gonadotrophin, in this case occurring before the expected menses, is that given by Levin (169). Pregnancy supervened in a woman in whom the variations of the gonadotrophic titer were being followed through the cycle, the titer varying from less than 5 to 15 MUU/day. The titer began to rise on the 24th day of the cycle; by the 30th day (of a 30-day cycle) 100 times as much was being excreted daily as during the normal cycle; 12 days later there was 3000 times as much as under non-pregnant conditions (see Fig. 5).

In two other cases (unpublished) one of us was able to observe the qualitative change from pituitary to chorionic gonadotrophin in one case between the 27th and 32nd day, in the other case between the 32nd and 36th day after the onset of the last menstruation. In the two instances, the lowering of body temperature (taken daily) indicated ovulation as having occurred in one case on the 17th and in the other on the 14th day and the change had hence occurred in one case by the 15th and in the other by the 22nd day following ovulation. The three cases appear to establish the remarkable fact that human chorionic gonadotrophin has already begun to appear in the urine between the tenth and twenty-second day after ovulation or between the fourth day preceding and the eighth day following the first missed menstruation. Further study will probably not push the day of its appearance much earlier than the tenth day after ovulation although the human blastocyst begins erosion of the maternal endometrium on the sixth day and the trophoblast has been detected entering a maternal sinusoid on the ninth day (Rock and Hertig, 217).

#### 6. *Titer of Human Chorionic Gonadotrophin during the Course of Gestation*

Zondek (Aschheim and Zondek, 10 and Zondek, 280) described the titer of hormone as rising abruptly early in pregnancy, reaching a maximum within the first few weeks, remaining constant for the rest of gestation, falling slightly, especially in the last couple of months, so that at the end of pregnancy the hormone sometimes could not be detected in native urine. The actual unitages cited are shown in Table VI. After parturition the titer was found to fall rapidly, being back to the normal level by the 8th day of the puerperium.

TABLE VI  
GONADOTROPHINS IN URINE OF PREGNANT WOMEN  
TIME OF APPEARANCE OF INCREASED GONADOTROPHIN IN URINE; THE PEAK AND RECESSION FOUND TIMED FROM LAST MENSTRUATION

Author	Early			Maximum ("Peak")			Late		
	Time	Titer	Time	Time	Titer	Time	Titer		
Aschheim and Zondek, 1927, 1928	35-42 40-80	Appears 3000-5000 MU/li	3-7 mo.	3,000-6,000		7-10 mo.	2000-3000		
Aschheim, 1935	28								
Zondek, 1935	28		1-8 wk. 3-7 mo.	5,000-30,000 MU/li 5,000-16,000 MU/li		7-10 mo.	4000-12,000		
Heim, 1935			3 mo.	166,000-250,000 MU/li					
Smith and Smith, 1935	30-60	100-2000 RU/24 hr.	90-120 days	6000-8000		120 + days	400-2000		
Brown and Venning, 1936	40-50		50-60 days	100,000-300,000 RU/li		60-85 days	Decreases		
						80 days	10,000		
						120 + days	3,000		
Evans, Kohls and Wonder, 1937			50-80 days (60 days usual)	75,000-1,000,000 RU/day		80 + days	10,000		
Boycott and Rowlands, 1938	42+	rapid rise	56-84 days			140 + days	Low titer constant level		

Levin, 1941	23	Appears			
	24	70 MUU/24 hr.			
	30	1,500 MUU/24 hr.			
	42	40,000 MUU/24 hr.			
Fosco, 1943	25-30	Appears	50-60 days (duration 1-3 wk.)	20,000-40,000 MUU/day	100-120 days 1000-5000
Seeger-Jones, Delfs, and Stran, 1944	28 + days	5000 IU/li	40-60 days	70,000-100,000 IU/li	100 days 130 + days 10,000 5,000

Heim (127) described much higher titers during the 3rd month of pregnancy—166,000 and 250,000 MU/li respectively.

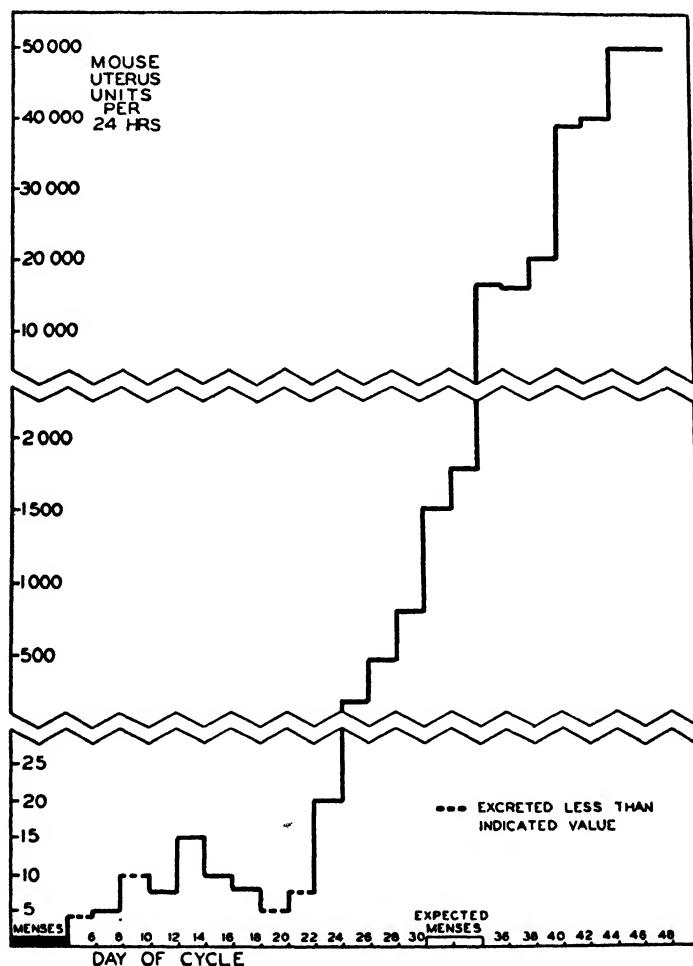


FIG. 5.—Sudden rise in human urinary gonadotrophin detected early in a single case of pregnancy. (After Levin, 169.)

Browne and Venning (22), described a marked increase in concentration beginning 40 to 50 days from the last experienced menses and reaching a peak of 100,000–300,000 RU/li between the 50th and 60th day, the decline began between the 60th and 85th day; less than 30,000 RU were present after the 120th day, and less than 10,000 RU the last 200 days of pregnancy.

Evans, Kohls, and Wonder (72) described a sharp peak between the 20th and 50th day from the last missed period at which time 75,000 to 1,000,000 RU were excreted during 24 hours. Less than 10,000 RU were excreted daily during the last 200 days (Fig. 6).

The authors cited in Table VI, and many others, agree that there is a rapid rise in the titer early in pregnancy appearing at the time or slightly before the first missed period and reaching a peak of secretion around the 50th and 60th day from the last known menstruation, then rapidly diminishing. There are still differences in determination of the duration of the greatly elevated excretion and in the normal limits of variation in titer. As far as can be judged, the peak of secretion lasts about three weeks.

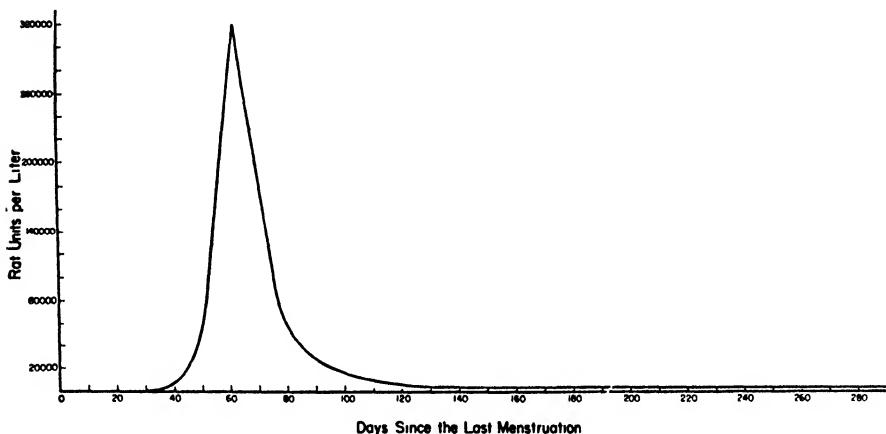


FIG. 6.—Schematic curve of the titer of human urinary chorionic gonadotrophin in normal pregnancy; there is shown the time of occurrence of the extremely rapid rise and fall (the, "peak phenomenon" in this titer. (After Evans, Kohls, and Wonder, 72.)

Fosco (93) in summarizing considers that 20,000–40,000 MU/24 hour is a better average figure for the maximum rather than the higher values reported. The decrease in concentration is almost as rapid as the increase. There is agreement that the excretion late in pregnancy is relatively low, and that the hormone disappears from the urine by 5–10 days post partum.

#### 7. Titer of Human Chorionic Gonadotrophin in Abnormalities of Pregnancy

Increased hormone outpour is reported associated with deranged or persisting placental tissue. It has already been shown that the very high hormone production originally reported by Zondek as characterizing hydatidiform mole and chorioepitheliomata is characteristic for a very short period of normal pregnancies—the early period characterized as the "peak"

of excretion. Where such high values are found late in pregnancy we must look for a mole or its transformation products.

A dramatically sudden drop and disappearance of chorionic gonadotrophin from blood and urine occurs after parturition. The persistence of high hormone values in later pregnancy, or in the puerperium, or an increase in output after a decrease has occurred, constitute valuable diagnostic evidence of deranged placental function. Values of 200 to 300,000 MUU/liter are currently accepted (Mathieu, 188; Fosco, 93) as indicative of abnormality of the placenta, or in the beginning puerperium, the indications are that chorionic tissue has been retained.

The characteristic transformation of the chorion known as hydatidiform mole is invariably associated with a greatly increased secretion of gonadotrophin and there is the same dramatic decrease of HCG in the urine after passage of a mole as occurs at parturition. The well known origin of chorioepithelioma from retained mole fragments has made it mandatory in the clinical history of a passed mole to examine the urinary titer of HCG for at least two or three fortnightly intervals and subsequently when indicated. The establishment of a chorioepithelioma is spectacularly evidenced by a return of the high unitage and a climb to greater heights than is known in any other circumstance; unitages of a million RU per liter in blood and urine may ultimately occur.

It is of the deepest interest that typical chorioepithelioma may also occur in the male where it is usually a neoplasm of the testis. This experience has supported the concept that HCG is normally elaborated by the Langhans layer of the chorion and has neither a pituitary nor an ovarian source. (See Section on hormonal excretion in men with testicular tumors.)

The syndrome hydrops fetus universalis et placentae, or fetal erythroblastosis, has recently (Herrnberger, 134 and Zsigmond, 285) been described as characterized by increased hormone titers. Rupp (225) finds the high titers only in those instances characterized by edema of the placenta—a not invariable accompaniment of the syndrome.

Increased gonadotrophic titers in pregnancy have been reported in the toxemias of late pregnancy, pre-eclampsia and eclampsia (Smith and Smith, 1948 (244).

Seegar-Jones, Delfs, and Stran (233) also Fosco (93) find that multiple pregnancies are associated with increased titers. The explanation given was the larger volume of placental tissue. The same explanation is offered by Fosco for the increased excretion in hydatidiform mole and in chorioepithelioma.

#### B. GONADOTROPHIC HORMONE IN THE URINE OF NORMAL WOMEN

The first analyses of normal adult women's urine for gonadotrophin were negative, as the volumes of native urine which could be injected were not

adequate to show the low unitage now known to be present. Alcohol concentration methods yielded concentrates difficult to test due to their toxicity, and methods of detoxification had to be devised before satisfactory titers or unitage could be obtained. Washing with alcohol and ether, and dialysis have been the standard methods for removal of toxic substance. The tungstic acid precipitation methods of Katzman and Doisy (150) yielded non-toxic preparations if used properly. Tannic acid precipitation was introduced by Levin and Tyndale (171), for the concentration of this type of urine. Gorbman (106) introduced a simple ultra filtration method which has been carried out extensively by Heller *et al.* (see Jungck *et al.* (147)). Bradbury *et al.* (19) have suggested concentration by kaolin adsorption.

By the application of concentration methods, gonadotrophin was first detected in the urine and blood during the middle of the menstrual cycle, later it was shown that this is a period of increased excretion and that lower rates of excretion characterize the rest of the cycle.

The unitages reported as occurring in normal women vary widely but a comparison of the results of different workers is difficult as many different assay methods have unfortunately been employed.<sup>8</sup> There is no international standard available to compare the various types of response. Another difficulty in comparison of results arises from expressing given results in some instances in terms of daily output and in others in units per liter of urine. Further, it is doubtful whether concentration methods used give uniformly quantitative results. Even with a given biological testing method, variability in animal response introduces wide fluctuations in results, especially where lower unitage urines are being tested, e.g., excretions of 3-6 units per day, where one is often limited in test material and hence in the number of test animals which can be used in the assay. The unsatisfactory state of our knowledge is evident from Table VII.

One thing clear from all assays is that the unitage excreted by normal women is very low, usually near the limits detectable by the methods which have been used, (3 to 6 units) except at the time of the peak of excretion in the mid-menstruum. Here the concentration varies from 10 to 50 mouse uterine units, above 50 units being definitely high.

<sup>8</sup> Changes in mouse and rat ovaries were first used. The mouse uterine unit was introduced by Levin and Tyndale (172), as more sensitive, three times more sensitive than the ovarian response. The rat uterine response is also used (Heller, Lauson and Sevringshaus (129)). Katzman and Doisy used vaginal opening and cornification in the rat as the basis of their unit. Others plotted uterine or ovarian weights directly as the measure of gonadotrophin output at different times of the cycle without interpreting the response in terms of units (Heller, Farney, Morgan, and Myers (131)). These results are especially difficult to compare with those in which units are used.

**TABLE VII**  
**GONADOTROPHIC TITERS IN URINE OF NORMAL (YOUNG) WOMEN**

Author	Unit	Minimum Unitage*	Maximum	
			Unitage	Time in Cycle—day
Zondek, 1931	Ovarian	5 RU/liter	25 RU/liter	Mid-cycle pre-menstrual
Katzman and Doisy, 1933	Estrus (vagina and uterus)	3 MU/day	16 MU/24 hr.	
Katzman and Doisy, 1934	Estrus (vagina and uterus)	3 MU/day	31 MU/24 hr.	
Frank and collabo- rators, 1935	Rat ovarian	2 RU/liter	2 to 25 RU/liter	10-14
Frank, Goldberger, Salmon, Felshin, 1937	Rat ovarian	2 RU/day	60 RU/day	10-14
Kurzrok, Kirkman and Creedman, 1934	Rat ovarian	3 RU/liter	25 RU/liter	11-14
Rothermich and Folk, 1940	Mouse uterus increase 100-150%		30 MUU/day	
Haam and Rother- mich, 1940	Mouse uterus increase 100-150%	5 MUU/day	45 MUU/day	10-16 most constant
Levin, 1941	Mouse uterus increase 100-150%	7.5 MUU/day	10-35 MUU/day	12-13 20-21
Werner, 1941	Mouse uterus increase 100-150%	5 MUU/day	15-80 MUU/day	9-18
Klinefelter, Al- bright, and Gris- wold, 1943	Mouse uterus increase 100-150%	6 MUU/day	53 MUU/day	11-13

Many have thought that the mid-menstrual peak coincides with ovulation or just precedes it. Peaks of excretion have, however, not been found to be confined to the mid-menstruum. Some workers have observed two peaks, others have reported a multiplicity of peaks and have been unable to determine a consistent time of occurrence of these peaks (Heller *et al.*, 130; Werner, 275; Main *et al.*, 182). On discovery of the secondary peaks it became problematical whether increased excretion could be used to mark the time of ovulation.

The reported constancy of the relation between the estrin and gonadotrophin peaks convinced many workers that there was a causal relation between them. Werner (275) found, however, that the estrogen peak may occur before, with, or after the peak of gonadotrophin output. More commonly, the mid-menstrual peak of estrin excretion has been found to precede the mid-menstrual peak of gonadotrophin excretion by one to two days (D'Amour, 51; D'Amour and Woods, 52; D'Amour, 53; Haam and Rothermich, 118). The second well recognized peak of estrin excretion (in the last part of the cycle or premenstrually) does not always correspond to a secondary peak of gonadotropin.

Heller, Farney, Morgan, and Myers (131) attempted to see if a correlation existed between the ovarian and endometrial histology, vaginal epithelium, gonadotrophic hormonal excretion and the day of the menstrual cycle. They found that though the gonadotrophic assay could be used to distinguish an atrophic from an active, functioning ovary, it could not be used to differentiate the specific functional state of the ovary; the uterine samples gave a better index. Since then, Venning and Browne (268) have definitely determined that pregnanediol appears in the urine 24 to 48 hours after ovulation.

The Venning and Browne urinary pregnanediol determinations have been made in an effort to check the significance of the gonadotrophin-estrin peak in relation to ovulation (Werner (275), D'Amour (53). Pregnanediol excretion is obtained slightly after that peak.

The biological characteristics of the hormone excreted by women during the normal cycle have received some consideration, although not as much as the gonadotrophin excreted in the menopause or after castration or even by normal men, probably due to the low excretion rate characteristic of normal women except during the peak periods. The biological effect as first observed by Zondek was follicle stimulation only. Several workers have since noted luteinization on prolonged administration or at high doses (Frank, Goldberger, and Spielman, 99; D'Amour, 51; Salmon and Frank, 228). The commonly accepted opinion is that the hormone which occurs in the blood and urine of normal women throughout the cycle is predominantly follicle stimulating, resembling that in the urine of castrate or

menopausal women, and that the hormone is probably derived from the pituitary.

### C. MENOPAUSE (CASTRATION) URINARY GONADOTROPHIN

Shortly after the discovery of urinary chorionic gonadotrophin by Aschheim and Zondek, Fluhmann (87,88,89,90) detected a gonadotrophic substance in the blood of women during the menopause epoch; shortly afterwards Zondek (279) reported a similar substance in the urine after the menopause. The same urinary material was found after castration of

TABLE VIII  
GONADOTROPHIC TITERS IN URINE AFTER CASTRATION AND AFTER THE MENOPAUSE

Author	Unit	Unitage	
		Minimum	Maximum
Katzman and Doisy, 1934	Estrus (uterus and vagina)	9 MU/day	26 MU/day
Katzman, 1937	100% uterine increase	7.4 RU/liter	16 RU/liter
Salmon and Frank, 1935	Ovarian development	10 RU/liter	40 RU/liter
Levin and Tyndale, 1935	200% uterine increase	70 MUU/liter	80 MUU/liter
Rothermich and Folk, 1940*	100% uterine increase	50 MUU/day	100 MUU/liter
Heller and Heller, 1939	Uterine stimulation	33 RU/liter	100 RU/liter

\* *Endocrinology* 27, 87 (1940).

either men or women. The concentration of material is so slight that relatively large amounts of urine must be handled to obtain any appreciable recovery of the substance and this has not hitherto been done on any extensive scale. For these reasons neither careful biological experimentation nor physicochemical studies have been made on the substance. It has been generally assumed that it is of pituitary origin.

Titers of gonadotrophin in castrate and menopause urine are reported to vary between 50 and 100 MUU/24 hours. (See Table VIII.) The titer rises 6 to 10 days after castration and is maintained (Heller, Farney, Mor-

gan and Myers (131). In contrast to chorionic gonadotrophin this material, when administered to normal immature rodents, does not typically induce the final ripening of ovarian follicles with subsequent ovulation and corpus luteum formation. Its predominant effect is as a stimulant to follicular growth. In hypophysectomized rats the contrast with chorionic gonadotrophin is even more marked. It will be remembered that the latter substance brought about the enlargement of the thecal and interstitial cells but was incapable of producing growth of the ovarian follicles. The castration type of gonadotrophin initiates growth of multiple follicles without significant effect on the thecal and interstitial tissue. These findings led to the view that the material was predominantly, if not exclusively, a follicle stimulating hormone, and it was frequently identified with pituitary FSH. As in the case of pituitary FSH, the enlarged ovarian follicles produced in hypophysectomized animals did not attain maturity but when the material was combined with chorionic gonadotrophin the mixture did ripen and ovulation and corpus luteum formation resulted (synergism) (Leonard and Smith, 168).

In the hypophysectomized male rat, the seminiferous epithelium is preserved from atrophy or restored without appreciable effect on the Leydig tissue and the accessory organs of reproduction.

The foregoing statements characterize the chief attributes of the hormone. It has been shown, however, that variable amounts, usually small amounts, of interstitial cell stimulating substance are always present in the blood and urine of human beings in the menopause or after castration. At high doses, interstitial cell repair results in hypophysectomized rats, and corpora lutea are formed in normal immature rats. The hormone from this source therefore has the gonadotrophic characteristics of pituitary hormone; it is similar to the hormone in the castrate pituitary where the follicle stimulating effects greatly predominate. Although the assumption is commonly made that this hormone found in the blood and urine is derived from the pituitary no one has chemically fractionated it into two gonadotrophins like FSH and ICSH. There is good experimental evidence that the castrate pituitary produces and secretes into the blood stream increased amounts of a gonadotrophin, which is predominately follicle stimulating in effect. The increased gonadotrophic content of the castrate rat's pituitary was first shown by the implant method. Experiments with parabiotic rats, a castrate male or female united parabiotically with a normal or hypophysectomized female, show that the castrate animal's pituitary is pouring increased amounts of a follicle stimulating hormone into the blood stream as the parabiotic partner of the castrate shows excessive follicular development and continuous estrus. Interstitial cell stimulating substances are, however, present and secreted in

small amounts as can be shown by the seminal vesicle and prostate response of hypophysectomized males connected parabiotically with such castrates (Greep, 108).

#### D. URINARY GONADOTROPHIN IN CHILDREN

The earlier method of concentration of urine and the tests used were not adequate to detect gonadotrophic hormone in the urine of children. Katzman and Doisy (150) used a method of tungstic acid precipitation sufficiently sensitive to detect 2.4 to 3.7 RU per 24 hours. They reported that prepubertal boys and girls did not excrete detectable amounts of gonadotrophin. Small amounts of hormone were detected in a few instances in post-pubertal girls and boys, 4 RU (16 MU) and 7 RU (28 MU) respectively.

TABLE IX  
GONADOTROPHIC TITERS IN URINE OF CHILDREN

Author	Unit	Unitage
Katzman and Doisy, 1933, 1934	Estrus vagina and uterus	2.8-4.7 MU/day
Catchpole, Greulich, and Sollenberger, 1938	Uterine weight increase	2-3 MUU/day

Catchpole *et al.* (31), and Greulich *et al.* (112) were unable to detect 3 MUU per 24 hours of urine in children up to 12 years of age, positive tests being first obtained between 13 and 14 years. After 14 years the value definitely increased toward the values for normal adults. (The normal range in men as found by their methods was 7 to 20 MUU/24 hours.) They used a modified tungstic acid precipitation method and the unit was defined as the dose which gave an increase in the weight of the mouse uterus given by 0.1 IU of human chorionic gonadotrophin. They conclude from their observations, and those reported in the literature that excretion of gonadotrophin in early childhood in both sexes is very low, being below the range dependably detectable by current methods of concentration until adolescence. (See Table IX.)

The conclusions of Nathanson, Towne, and Aub (197) from the use of alcohol precipitation were essentially the same. In boys follicle stimulating substances were not detected before 13 years; in girls positive findings appeared during the 11th year.

### E. URINARY GONADOTROPHIN IN NORMAL MEN

Excretion of gonadotrophic hormone in urine cannot be detected in normal men unless the urine is concentrated. By use of alcohol precipitation it became possible to inject the equivalent of a much larger volume of urine and workers using it began to describe hormone in the urine of normal men. Such preparations contained so much toxic material that many discarded the alcohol preparation method. In its place tungstic acid, benzoic acid and tannic acid precipitation methods were recommended. Many laboratories have since returned to alcohol precipitation methods, modified to reduce toxicity by such procedures as alcohol and ether detoxification, dialysis or fractionation with alcohol (extraction with 50% alcohol and reprecipitation in 80% alcohol).

Though normal male urine is often cited as being purely follicle stimulating, most workers have found that at higher doses, as in the case of menopause urinary hormone, interstitial tissue repair and corpus luteum formation occur, (Harris and Brand, 124; Starkey and Leathem, 257; Leathem *et al.*, 164, 165; Seegar-Jones and Bucher, 231). Like menopause urine, and unlike HCG, it produces marked ovarian enlargement. In male rats it stimulates the germinal epithelium but has very little effect on the Leydig tissue and accessories. Some have concluded that the urine contains an almost pure follicle stimulating hormone with only a trace of interstitial cell stimulating or luteinizing properties. Fraenkel-Conrat, Li, Simpson, and Evans (95), also Evans and Gorbman (77) have titrated the ratio of follicle stimulating and interstitial stimulating properties in hypophysectomized female rats and have found the ratio to be two (or three) to one.

Evans, Simpson, Tolksdorf, and Jensen (73) and Fraenkel-Conrat, Li, Simpson and Evans found that like other non-pituitary gonadotrophic substances this hormone from male urine did not antagonize the action of other gonadotrophins when it was injected intraperitoneally.

Fraenkel-Conrat, Li, Simpson and Evans postulated that male urinary hormone must produce part of its effects in the normal animal by stimulating the pituitary. The basis for the conjecture was that the substance was effective in hypophysectomized rats only at a multiple of the dose necessary for gonadotrophic effects in the normal animal, the ratio of potency in animals with and without a pituitary being the same for all non-pituitary gonadotrophins with interstitial cell stimulating properties.

Fairly good agreement has been reached on the unitage normal for adult men. The titer definitely averages higher than in normal women. Considerable variation appears to exist between individuals and from time to time in the same person. The range reported is 7 to 120 MUU/24 hours.

(see Table X). In most instances the range lies between 20 and 40 MUU/24 hours (Klinefelter, Albright, and Griswold, 156). (Most workers

TABLE X  
GONADOTROPHIC TITERS IN URINE OF NORMAL MEN

Author	Unit	Unitage		
		Minimum	Maximum	Average
Katzman and Doisy, 1933, 1934	Estrus vagina and uterus	4 MU/da	19 MU/da	7 RU (28 MUU)
Katzman, 1937	Vagina open, estrus	2 RU/liter	4.7 RU/liter	
Saethre, 1935		11 MU/da	38 MU/da	28.4 MU/da
Levin, 1941	150% uterus	10 MUU/da	25 MUU/da	
Heller, Heller and Sevrinhaus, 1941		2.5 MUU/da	10 MUU/da	
Evans and Gorbman, 1942	Mouse 100%, uterus increase Rat, IF, estrus	6 MUU/liter 1 RU/liter	20 MUU/liter 4.5 RU/liter	
Catchpole, 1942 (in Gruelich, Dorfman, Catchpole, Sherman and Culotta)		7 MUU/da	20 MUU/da	10 MUU/da
Werner, 1943	100-150%, uterus increase	5 MUU/da	120 MUU/da	40 MUU/da
Klinefelter, Albright and Griswold, 1943		7-13 MUU/da	53-105 MUU/da	13-26 MUU/da

have used the mouse uterine test in such standardizations because smaller unitages are detectable than by the rat ovarian test.)

Though an increase in gonadotrophic titer in old men has been reported, the use of improved methods shows no marked or constant increase in titer

in men comparable to that seen in women. This is associated with the more prolonged fertility and the more gradual disappearance of gonadal function in men than in women. Periodicity in excretion rates has been described but the results are preferably interpreted as non-rhythmic variations.

#### F. URINARY CHORIONIC GONADOTROPHIN IN TUMORS OF THE TESTIS

A vast amount of effort has been expended by urologists and capable pathologists in a satisfactory classification of the histological types of tumors of the testis, and, during the last few years efforts have been made to correlate urinary gonadotrophin findings with tumor types. We have already mentioned the occurrence of low titers of urinary-gonadotrophin in normal men. This is apparently of pituitary origin and possesses both follicle stimulating and interstitial cell stimulating potency. It has also been shown that gonadotrophin is invariably elevated in both blood stream and urine after castration. The claim that some types of testicular neoplasm are characteristically associated with increased amounts of gonadotrophin of true pituitary origin (Hamburger) has not yet been satisfactorily substantiated. On the other hand, widespread agreement exists as to the absence of appreciable amounts of urinary gonadotrophin in testicular teratomata consisting of so-called adult or differentiated tissues (epithelium, hair, nails, cartilage, etc.) There appears also to be a similar absence of urinary gonadotrophin in the neoplasm designated as seminoma or the Chevasu tumor. Finally, there is also clarity and unanimity regarding the association of chorioepitheliomata with an extraordinarily high titer of urinary chorionic gonadotrophin. So perfect is this correlation and so invariable the findings, that one can diagnose this neoplasm from urinary hormonal titrations in those rare but now well-known instances of the occurrence of this neoplasm away from the testis itself, apparently from displaced testicular tissue, for example in a retroperitoneal location. This experience, indeed, constitutes excellent confirmation of the contention that chorionic gonadotrophin is secreted by the epithelial component of choriomatous tissue. The finding of as high a titer as 1000 RU per liter of urinary gonadotrophin itself indicates the presence of choriomatous tissue and the prognosis of fatal malignancy. The approach of the exitus lethalis may be heralded by an extraordinary rise in the hormone titer of blood and urine, and unitages of a million units per liter in body fluids may be encountered at autopsy.

There is still controversy regarding the existence of some testicular neoplasms of double or multiple nature, in which the chorionic component, though slight, metastasizes readily. This type appears to be established by the finding of small chorionic components in a tumor consisting other-

wise almost exclusively of typical seminoma cells or being a typical embryonal carcinoma, the chorionic component only being detected by serial section of large portions of the original neoplasm.

TABLE XI  
SUMMARY OF THE USUAL GONADOTROPHIC HORMONE TITERS IN HUMAN URINE

Type of case	Urinary hormone titer
Children	2 or 3 MUU/day
Normal Woman	
Limits	5-50 MUU/day
Mid-cycle peak	20-50 MUU/day
Early and late in menstrual cycle	5-7 MUU/day
Pregnancy	
Early—first trimester	30,000 to 1,000,000 RU/day
Late	2,000 to 8,000 RU/day
Hydatidiform mole and chorioepithelioma	>1,000 to 1,000,000 RU/liter
Normal men	
Limits	7-105 MUU/day (Av. 20-40)
Average	
Testis tumor: (chorioepithelioma)	1,000 to 1,000,000 RU/liter
Castration or menopause	50-300 MUU/liter
Hypogonadism	
Pituitary origin	Below normal: 3 or 7 MUU/day
Gonadal origin	Normal or above normal: limits 50 to 200 MUU/day

#### G. OTHER CLINICAL CONDITIONS IN WHICH URINARY GONADOTROPHIN ASSAYS ARE ALTERED

The urinary gonadotrophin titers in normal individuals, men, women and children, have been summarized, as have the increased titers encountered in normal pregnancy, disturbances of pregnancy, hydrops feti, tumors associated with pregnancy, hydatidiform mole and chorioepithelioma, and similar tumors in men, associated with the testis, and in castration and menopause (see Table XI). There has been considerable interest in the extension of the field of clinical usefulness of gonadotrophin assays for determination of increased, or decreased, excretion in various syndromes, for example in distinguishing between hypogonadism primary in the gonad from

that secondary to pituitary hypofunction. The former group, primary hypogonadism, is supposed to be distinguished by increased titers of gonadotrophin in the urine. Ovarian agenesis (Turner), a condition characterized by congenitally aplastic ovaries and high gonadotrophic titer can be placed in this category (Albright, Smith, and Fraser (1), Varney, Kenyon and Koch (267), Lissner, Curtis, Escamilla, and Goldberg (177), del Castillo, de la Balze, and Argonz (54)). In the second type of hypogonadism, hypopituitary in origin, less than 3 or 6 MUU may be expected per day. In a syndrome described by Klinefelter, Reifenstein and Albright (155) characterized by hypogonadism, no spermatogenesis, but Leydig cells being present and associated with gynecomastia, the urinary gonadotrophin is elevated. In a similar syndrome (save for the absence of gynecomastia) del Castillo, Trabuco, and de la Balze (55), the gonadotrophic titer was not raised. Since Sertoli cells were abundant, the last mentioned authors were led to the hypothesis that the Sertoli cells are the source of a hormone which depresses pituitary gonadotrophin output.<sup>4</sup>

#### IV. Hormonal Requirements for the Maintenance of Pregnancy

Smith (253) showed that the rhesus monkey may be hypophysectomized as early as the 32nd and as late as the 106th day of pregnancy and yet will complete gestation with the birth of living young although incapable of lactation.

The anterior pituitary is essential for the establishment and maintenance of pregnancy in the rat to the 11th day. After this time the pituitary can be removed and gestation will continue. (Pencharz and Long, 204). Pregnancy can be maintained after nidation has occurred or can even be established, using a relatively crude lactogenic hormone (Cutuly, 48, 49, 50). Lyons, Simpson, and Evans (180) maintained pregnancy with a preparation of lactogenic which was not entirely pure, but were unable to do this with pure lactogenic hormone. A small dose of estrogen was given simultaneously in the latter studies.

Lyons in 1943 (179) showed that pregnancy could be maintained in oophorectomized-hypophysectomized rats by estrin and progesterone, (1 microgram and 2 mg., respectively). As the ovaries are necessary in the rat throughout pregnancy it is assumed that in the latter part of pregnancy—after the 12th day—the placenta must produce a luteotropic

<sup>4</sup> This may well be estrogen, for Huggins and Moulder (144) discovered estrogen production by Sertoli cell tumors of the testis in the dog. Now Teilum (261) has brilliantly homologized certain ovarian and testicular tumors which may be called arrhenoblastomas; he has shown that the hormone-producing androblastomas may have a dominance of Sertoli (estrogen producing) or of Leydig (androgen producing) cells.

principle. A luteotrophic principle in the placenta was demonstrated by Astwood (13), and has been confirmed by Sussman (260).<sup>5</sup>

## V. Equine Gonadotrophin or Pregnant Mare Serum Gonadotrophin (PMS)

### A. DISCOVERY

In 1930 Cole and Hart discovered that the blood stream of pregnant mares contains a powerful gonadotrophic hormone. Later study (Cole and Saunders (39) has shown that the hormone appears at the 37th to the 43rd day of pregnancy when 60–100 RU per liter are present (implantation occurs in the mare at this period). It increases rapidly in amount until the 50th day of pregnancy when 50,000 RU per liter are present. The "peak" lasts until the 100th day of pregnancy and the unitage in the subsequent course of gestation falls gradually until less than 50 units are

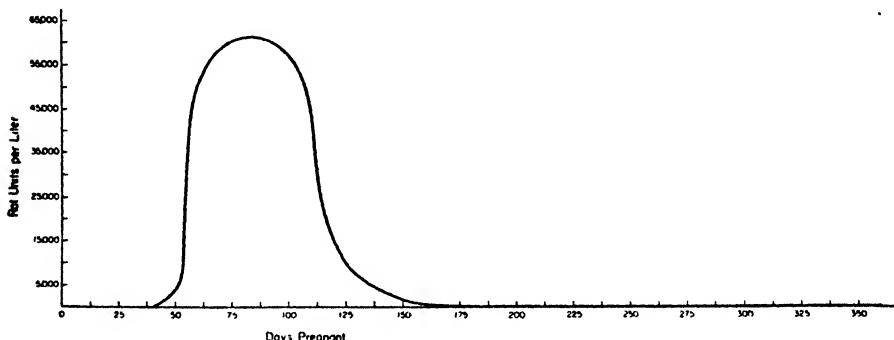


FIG. 7.—The concentration of gonad-stimulating hormone in the blood serum of a mare (C8) during pregnancy. (Modified after Cole and Saunders, 39.)

present on the 208th day (see Fig. 7). Cole (36) has made the very remarkable finding that the concentration in some ponies is higher than in larger equine breeds.

### B. SITE OF ORIGIN

The hormone was at first thought to be a pituitary hormone but the pituitary of the mare offers little histological or physiological evidence of increased activity. Large amounts of the hormone are present in the endometrium of the fertile horn. Catchpole and Lyons, (28,29), were inclined to the belief that the hormone, like that in human pregnancy,

<sup>5</sup> Although the studies of Astwood and of Sussman did not reveal crop sac stimulating potency in the rat's placenta, recent findings in our laboratory (Lyons, Averill and Ray, unpublished) indicate that both extracts and implants of the twelve-day rat placenta do definitely exhibit some crop sac stimulating as well as pronounced luteotrophic properties.

originates in the chorion. In one mare, they detected the hormone in the chorion before it was found in the blood stream or endometrium. Further, they found that endometrium in contact with chorion was richer in gonadotrophic activity than endometrium of the infertile horn. Cole and Goss (40) are convinced that the hormone is actually formed in remarkable cup-like endometrial structures (structures first described by Schauder in 1921) (Fig. 8). The content of these cups, a waxy substance, eventually makes a protuberance covered by the chorion. No decision was reached as to

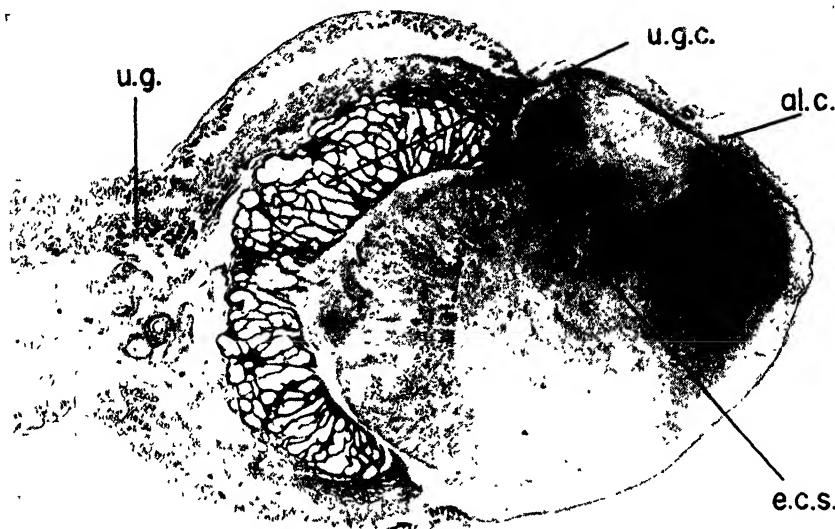


FIG. 8. Endometrial cup for mare No. 4, on the 105th day of pregnancy. u.g., uterine glands; u.g.c., uterine glands of the cup; al.c., allantochorion; e.c.s., endometrial cup secretion. (After Cole and Goss, 40.)

whether the uterine glands, which were distended with secretion in this region, were contributing the secretion, or whether the enlarged epithelioid stromal cells which are degenerating in this region, are responsible. Rowlands (223) is inclined to the view that the assumption of origin from the stromal cells most closely fits the observed relationships. Reservoirs of the hormone in enormous concentration are found free in the cups (300 IU per mg. of wet weight) and can be recovered by peeling off the endometrium itself.

### C. BIOLOGICAL PROPERTIES

It is remarkable that the hormone is not found in appreciable amounts in the urine of the pregnant mare or in that of animals to which it is admin-

istered, probably on account of its inability to pass the renal filter, for it can be recognized in the blood stream of the rabbit for many days (12 or more) following a single intravenous injection (Catchpole, Cole, and Pearson, 30). This phenomenon is in contrast with the speedy disappearance of human chorionic gonadotrophin from the circulation. In the latter case, while only a small portion of the injected hormone is recoverable in the urine, it nevertheless disappears rapidly from the blood stream due to an unknown method of degradation. The very slow disappearance of equine gonadotrophin is not influenced by ovariectomy. The long persistence in the blood stream of the administered hormone makes frequent readministration unnecessary.

In the normal immature animal, the hormone shows both follicle stimulation and corpus luteum formation. The predominant effect observed in hypophysectomized female rats is follicular development, but this is combined with abundant interstitial cell stimulation. Although some thecal luteinization may be present, normal ovulation and corpus luteum formation is difficult to induce. Seminal vesicles are abundantly stimulated in the hypophysectomized male and spermatozoan formation occurs (Evans, Pencharz, Simpson, and Meyer (68). The route of injection influences greatly the biological response. In hypophysectomized females the minimally detectable biological response to intraperitoneal injection is in the interstitial cells. The ovarian weights which can be induced in the immature rodent in a 72-96 hour test period by the hormone are very large and increase with the dosage.

That the equine gonadotrophin, like human chorionic gonadotrophin, stimulates the pituitary is indicated by the invariably larger ovarian response shown by normal immature, as contrasted with hypophysectomized, rats and by the greater frequency of corpus luteum formation in the normal. The minimal effective dose in normal rats is almost half the minimal effective dose in hypophysectomized rats, and the response in terms of ovarian weights is double in normal as compared with hypophysectomized rats.

Ovulation and superovulation can be induced in the immature rat at appropriate doses. Evans, Meyer, and Simpson (67) observed ovulation at doses greater than the minimal effective dose but less than 5 units. Cole (34) reported ovulation 72 hours after a single injection in the 25-day-old rat of 2 RU, higher doses being required for superovulation, the most favorable dose for superovulation and implantation of excess numbers of fetuses being 3-8 RU (Cole, 35). As many as 17 living fetuses were observed.<sup>6</sup>

<sup>6</sup> This happens to be the same number observed by Evans and Simpson (74) after injection of pituitary follicle stimulating hormone in combination with human chorionic gonadotrophin.

The equine gonadotrophin was concentrated by Goss and Cole (107) until 4,000 to 7,000 RU were present per mg. of solids; Rimington and Rowlands (211) reported preparations containing 12,500 IU/mg. Assuming 1 RU = 2 IU the unitages of the two preparations were equivalent. Li, Evans, and Wonder (173) showed that a preparation made by the method of Goss and Cole, containing 1000 RU/ing., behaved as a single protein, yet another preparation, made by Goss and Cole and testing 3500 IU/mg. was shown by Li not to behave as a single protein. The molecular weight of the Li, Evans, Wonder preparation was estimated as 30,000. The preparation contained carbohydrate. Like human chorionic gonadotrophin, and unlike the pituitary gonadotrophins, equine gonadotrophin has its carbohydrate content chiefly as galactose (ratio of hexose to hexosamine 2:1). This is an appropriate place to compare gonadotrophins from the three known sources in this respect (Gurin, 113) (Table XII).

TABLE XII  
COMPARISON OF CARBOHYDRATE CONTENT OF GONADOTROPHINS

Source of gonadotrophin	Hexose present		Hexose Hexosamine
	%		
Pregnant mare serum	Galactose	17.6	8.4
Human pregnancy urine	Galactose	10-12	5-6
Anterior pituitary: LII. FSH.	Mannose Mannose	2.8 4.5	2.2 4.4

Since equine gonadotrophin is a complete gonadotrophic stimulant in the sense that it exhibits both follicle-stimulating and interstitial-cell-stimulating properties, it was expected that it would constitute a most satisfactory therapeutic agent in types of amenorrhea, sexual infantilism etc., but the clinical experience hitherto accumulated has not yet given the hormone a satisfactory therapeutic province. Its employment to stimulate libido or potentia in men has been hindered by the impurity of preparations hitherto available and the well-known antigenicity of horse serum products (see section on anti-hormones).

Follicular development and estrous change in the sexual skin of *Macaca rhesus* have been observed following injection of the hormone (Engle and Hamburger, 64; Hamburger, 119, and Morse and Van Wagenen, 195). Hartman (125) induced a very low percent of monkeys (not ovulating spontaneously) to ovulate following injection of the hormone. Smith (251)

was able to maintain spermatozoan formation in male monkeys for 20 days, but repair was not complete when there was a lapse between operation and injection.

Several fields of usefulness of the equine hormone in veterinary medicine are suggested and may become established on confirmation of results already secured in domestic animals. Equine gonadotrophin is effective in stimulating estrus and ovulation in lactating sows after the 40th day of lactation, thus affording a means of concentrating the farrowing period (Cole and Hughes, 41). It will increase sexual activity in rams during the summer months; rams of certain mutton breeds are sexually inactive at this time (Cole, 33). Injected in ewes shortly before expected estrus, there is an increased incidence of twinning (Loginova and Lopyrin, 178). The use of equine gonadotrophin for the induction of estrus in mares has been very successful (Cameron, 26). Hammond's studies (122) also indicate that equine gonadotrophin may prove useful in the control of ovulation in cattle.

## VI. Factors Affecting the Response to Gonadotrophins

### A. SPECIES AND STRAIN DIFFERENCES

One of the most outstanding characteristics of hormones is their non-species specificity. The sheep pituitary gonadotrophin is for example effective in inducing precocious maturity in the rat. This principle of non-specificity does not however hold strictly true, in the sense that gonadotrophins are not equally effective in all *species*. Ovulation can be induced in rabbits at one-third the dose required for the mouse. (The body weight of the two species is therefore obviously not the predominating factor in determining sensitivity of response.) Riddle (209) first showed that the pigeon testis is extremely sensitive to stimulation by pituitary gonadotrophins, but does not respond to chorionic gonadotrophin (see also Evans and Simpson, 71). Even *strains* within species vary in their sensitivity to a given gonadotrophin. The immature males of the Sprague-Dawley strain of rats respond to ICSH with much greater seminal vesicle and prostate development than does the Long-Evans strain (Fraenkel-Conrat *et al.* (95) (see Table XIII). The discouraging results obtained in clinical gynecology in the employment of gonadotrophins of proven potency is an eloquent demonstration of the specificity of requirements of different animals. Species differences in hormonal requirements are possibly mirrored by the different proportions of FSH and ICSH extracted from pituitaries of different species; much more FSH can be obtained from the sheep or pig pituitary than from the beef anterior lobe.

The relative unresponsiveness of amphibia to mammalian hormones is no more striking than the differences in sensitivity of such closely related forms as rats and mice (see Table XIV).

## B. AGE AS AN INFLUENCE

The age of the animal not only influences the gonadotrophin content of the pituitary as already discussed, but secondarily, age affects the responsiveness of the gonads to various gonadotrophins; the response is now

TABLE XIII  
DIFFERING RESPONSE OF TWO STRAINS OF RATS TO THE PREPARATION  
OF ICSH

Strain	Total dose	Seminal vesicles	
		Weight	Increase above control
	mg.	mg.	%
Long-Evans	5.00	19	55
	0.50	13	6
	0.05	14	14
Sprague-Dawley	2.00	47	370
	1.00	38	280
	0.50	28	180
	0.25	22	120
	0.12	18	80

Assay by H. L. Fevold of ICSH prepared from Sheep Pituitaries cited in Fraenkel-Conrat, Li, Simpson, and Evans (95).

TABLE XIV  
Relative Sensitivity of 21-Day Mice and Rats\* (M/R) in 72 Hour Test to Various Gonadotrophins in Body Fluids

Hormone	Ratio M/R		
	Ovarian weight	Vaginal opening	Uterine weight
Menopause ..	12/1	3/1	5/1
Human chorionic .. .	1/1 to 3/1	>5/1	5/1
Equine gonadotrophin ..	5/1	>20/1	5/1

\* Rats 21 days of age are less responsive than the 24-25 day old rats.

known to be in some instances dependent upon the degree of maturity the gonads of the *young* animal had attained at the time of administration of the hormone. The ovary of the *old* animal is also reported to be less responsive to gonadotrophins, *e.g.*, the ovaries of women past the menopause (Kurzrok, 160) or the ovaries of old rats (Zondek).

### C. INFLUENCE OF ROUTE OF INJECTION

The *route of injection* selected for administration of gonadotrophins is also an important factor in the response elicited. For example, HCG, equine gonadotrophins and pituitary ICSH are effective at lower doses given intraperitoneally, whereas FSH is more potent when given subcutaneously. Or again, ICSH when given subcutaneously with FSH, augments its action; given intraperitoneally with simultaneously injected FSH (or other gonadotrophins) it minimizes (antagonizes) the gonadotrophic response to the FSH (see Table XV).

TABLE XV

#### EFFECT OF ROUTE OF ADMINISTRATION ON POTENCY OF GONADOTROPHINS

Substance	Minimum Effective Dose	
	Subcutaneous	Intraperitoneal
FSH	mg.	mg.
25		25 (50)
ICSH	50	12
PMS	2	1
HCG	5 (10)	5

After Evans, Simpson, Tolksdorf, and Jensen (73), Fraenkel-Conrat, Li, Simpson, and Evans (85) and Pencharz, *Proc. Soc. Exptl. Biol. Med.* 42, 525, 1939.

### D. DOSE IN RELATION TO BODY WEIGHT

It has not been found to be important to adjust dosage of gonadotrophins nicely to body weight. The differences in responsiveness of animals, such as rats, mice and rabbits appear not to be determined by body size but by other more important factors. The target organ seems to select the hormone efficiently regardless of the dilution factor. There is some evidence, however, that direct injection of hormone into the gonad gives responses at lower doses than does systemic injection (Zondek; Tuchmann-Duplessis).

### E. INFLUENCE OF THE NUMBER OF INJECTIONS

Equine gonadotrophin is almost as potent given as a *single* injection as when the same dose is given as *repeated* daily injections, Cole *et al.* (38). This is probably related to the fact that it is not excreted; it is certainly not found in the urine of pregnant mares nor in the urine of animals in-

jected with very large doses. Other hormones such as human chorionic gonadotrophin rapidly pass the renal filter.<sup>7</sup>

In spite of the fact that FSH and chorionic gonadotrophins injected in large amounts are lost in the urine there seems to be relatively little advantage to multiple injections.

## VII. Antigonadotrophins

### A. GENERAL

Collip and Anderson (44) were the first to study the decrease in the effectiveness of thyrotrophic hormone on prolonged administration. They were able to demonstrate the presence in the blood stream of the treated animals of a substance or substances which prevented the usual prompt response of previously untreated animals. It was perhaps unfortunate that they employed the term "anti-hormone" to designate the substance in question. Collip and co-workers soon also turned their attention to the effects of prolonged administration of gonadotrophins and demonstrated a similar diminished response in the reproductive tract on prolonged treatment. Substances that were "antigonadotrophic" were found in the sera of animals injected chronically with gonadotrophins.

Frequently the antisubstances, produced on the injection of a gonadotrophin from one species into another, also depress the response to other gonadotrophins. Conversely, human chorionic gonadotrophin given to man is not usually antigenic,<sup>8</sup> nor is equine gonadotrophin in horses. The gonadotrophin in the rat pituitary does not lose its potency on the prolonged implantation of rat pituitaries into rats, (Katzman, Wade, and Doisy, 151).

Sometimes the antigenadotrophic agent affects only one of the gonadotrophic potencies of the extract, e.g., suppresses its capacity to stimulate follicles when administered with the antisubstance, (Kupperman, Meyer, and McShan, 157).

There is good evidence that the chronic injection of gonadotrophins from

<sup>7</sup> Relatively little effort has been expended on the urinary recovery of injected gonadotrophins. Evans, Simpson, and Austin showed that pituitary gonadotrophin injected into *Macaca rhesus* could be found in the urine, Evans and Simpson (unpublished) *et seq.* Evans and Simpson (unpublished) have also shown that the urine of rats injected with sheep gonadotropin contained the hormone. The urine only showed follicle stimulating potency in hypophysectomized rats though both follicle stimulating and interstitial cell stimulating potencies were present in the material injected. (The urine therefore reacted biologically, more like castration or menopause urine.) Human chorionic gonadotrophin was also recovered in the urine of injected rats.

<sup>8</sup> Though this certainly can occur, Leathem and Bradbury (166).<sup>\*</sup>

another species may inhibit the action of endogenous hormones. Meyer and collaborators have shown that precocious maturity may result in young rats when injections are stopped. The pituitaries during the chronic injections show castration cell changes and after cessation of treatment revert back to normal morphology. It would appear that the pituitary production of gonadotrophin continued during chronic hormone administration but was unable to influence the end organ.

Progonadotrophins (augmentation effects) as well as antigonadotrophins, are reported on chronic injections of gonadotrophins; the production of pro-gonadotrophins frequently precedes anti-hormone formation, (Marvin and Meyer, 186).

The antigenic potency of some preparations, *e.g.*, those resulting from injection of preparations from pregnant mare serum, decreases with increased purity of the hormone, indicating that much of the work on the anti-gonadotrophic reaction has been premature in the sense that the results may have been due to contaminants and not to the protein hormone *per se*.<sup>9</sup>

The biological phenomena upon which interpretations of anti-gonadotrophic and pro-gonadotrophic activity have been based are complicated and still inadequately understood.

### B. POSSIBLE IDENTITY WITH ANTIBODIES?

Collip and co-workers were cautious from the first discovery not to identify the antisera with immune bodies.

Sulman (259), and Zondek and Sulman (281), were also conservative as to the antigenicity of HCG, because of their difficulty in demonstrating typical antibody reactions to injected purified HCG (*i.e.*, complement fixation, precipitins and skin reactions). In their general treatise on anti-gonadotrophins they were, however, willing to admit that anti-gonadotrophins were immune bodies but a new type since "they do not give the *in vitro* reactions which generally characterize an immune body."

### C. PROHORMONE REACTIONS

Collip (43), Rowlands (218), and Thompson (262), found that the sera from animals receiving gonadotrophic agents were sometimes agumenting rather than antagonistic to the hormone injected or to other gonadotrophic agents. Katzman, Wade, and Doisy (152) tried to rid themselves of confusing species specific proteins present as contaminants of the gonadotrophin injected by implanting rat pituitaries into rats. After nine months the ovaries of the recipients were still large and the serum did not

<sup>9</sup> The antagonistic substance present in late pregnancy in the mare is considered by Cole (37), and Cole and Hughes (41), to be estrogen.

give antihormone effects—even gave augmentation; this was, in fact, the first observation of a progonadotrophic serum.

Katzman, Wade, and Doisy also injected serum from sheep that had been injected 262 days with sheep pituitary preparations and observed pro-gonadotrophic rather than anti-gonadotrophic reactions. In 1947 Katzman, Wade, and Doisy (153) reported that sera from sheep injected with sheep pituitary extracts were pro-gonadotrophic or anti-gonadotrophic varying with the time after onset of injection, sera collected and method of injection of antisera (given in several small doses they were inhibitory, single injection being augmenting in action).

Marvin and Meyer (187), reported that the reaction from sera was frequently first augmenting, then antagonizing, the latter being quite non-specific (*e.g.*, when sheep pituitary was injected the serum inhibited sheep, rat, beef and human pituitary extracts; also PMS and prolactin, but did not antagonize horse, hog and chick pituitary extracts).

Thompson (262), also found that sera from animals injected with pituitary extracts were at first augmenting, then on continued injection became antagonizing.

Rowlands (220, 221), observed that serum of rabbits injected with ox pituitary inhibited selectively in immature rats the luteinizing activity of gelding pituitary extracts, follicle stimulation being the only effect (*i.e.*, unfractionated extracts containing FSH and LH injected with antisera gave FSH effect only, the LH effect being neutralized). Such evidence has been cited in support of the presence of two separate gonadotrophins in pituitary extracts.

Rowlands (222), interpreted the augmentation of gonadotrophic effects observed at certain stages of chronic injection as possibly due to suppression of normally predominant luteinization in the test animal (*i.e.*, in rat pituitary). (He observed that serum of goat injected with pig pituitary had strongest augmenting effect on this extract when tested in immature female rats after six weeks.) Another suggestion of pituitary involvement in the pro-gonadotrophic reactions is found in the 1940 work of Rowlands and Williams (224). They report here that serum from a goat injected with pig pituitary extract was pro-gonadotrophic in normal but not in hypophysectomized rats.

Kupperman, Meyer, and McShan (157), have reported antisera with selective FSH properties. FSH prepared by tryptic digestion of sheep pituitary extract, also crude extracts, were injected into rabbits. FSH and original extracts were then tested with sera of injected animals. Antisera for FSH inhibited gonadotrophic activity of FSH, PMS and original extracts in females, but had no inhibitory effect on LH (as indicated by continued response of male accessories). Antisera from injection of the

crude pituitary extracts inhibited LH, original extract, and PMS effects in male and female test animals.

#### D. NON-SPECIFICITY OF ANTAGONISM REACTIONS

Rowlands (220), observed neither complete species specificity nor source specificity in the anti-gonadotrophic effects elicited. Antisera of PU and PMS had complete species specificity; extracts of pituitary developed incomplete species specificity. Complete source specificity was noted in antisera obtained from the injection of PMS.

Thompson and Cushing (263), found that dogs injected chronically with sheep pituitary extracts showed various degrees of non-species specificity to different gonadotrophic agents. After seventeen months, the serum antagonized in rats the gonadotrophic action from any source, pituitary, pregnancy urine or pregnant mare serum. It also prevented the development of the ovaries of an injected pup—*i.e.*, antagonized endogenous hormone.

#### E. ANTAGONIZING ENDOGENOUS HORMONE—PITUITARY CHANGES WITH ANTISERA

Collip, Selye, and Williamson (45), had noted pituitary changes associated with antiserum production; when atrophy of the ovaries occurred signet ring changes in beta cells were noted.

Marvin and Meyer also concluded that in general anti-substances were not specific and could even inhibit endogenous gonadotrophin. Meyer and collaborators, (Kupperman Meyer, and Finerty, 158; Meyer, Kupperman, and Finerty, 192, Finerty, Kupperman, and Meyer, 85; and Finerty, Meyer, and Marvin, 86), also observed castration-like changes in pituitaries of animals where antihormone effects were present and ovaries were atrophic. Precocious maturity might follow cessation of injection in young animals. The pituitary came back to normal. The interpretation was that the end organ had been prevented from reacting and the pituitary, though increased in hormone content as after castration, could not affect it.

The end organ may react to the gonadotrope injected even though anti-gonadotrophins are present. Freud and Uyldert (101), found that male rats showed no loss of gonad or accessory organ weight when injected daily for 56 days with CG, PMS or horse pituitary gonadotrophin. Their sera antagonized these gonadotrophins in immature male and female rats. When gonadotrophic treatment of normal or hypophysectomized male rats is interrupted, the accessory organs regress, showing that the gonadotrophin was acting although the serum contained anti-gonadotrophic activity. (Antihormone production in the male as in the female had been reported

by Collip—the response of the reproductive system of the male on the whole paralleling that of the female.) Marvin and Meyer (186, 187), had also observed the same pituitary changes in the male as in the female.

The results of Kupperman and Meyer (159), with parabiotic triplets could be interpreted as showing peripheral neutralization of endogenous gonadotrophin. Two normal females were placed on either side of a castrate male. One female injected with antiserum showed inhibition to the gonadotropic effects from the castrate male, whereas the ovaries of the female united on the other side of the male, reacted vigorously to the increased output of gonadotrophin characteristic of the castrate pituitary. The interpretation is that the circulating endogenous gonadotrophin was neutralized in the one female injected with antiserum—the female united parabiotically on the other side did not come under the influence of the anti-hormone.

#### F. DISAPPEARANCE OF ANTIGONADOTROPHIC EFFECTS AFTER INJECTIONS CEASE

The result described by Meyer and co-workers, in which precocious puberty occurred after ceasing injection, illustrate that anti-gonadotrophic effects disappear after injection of the gonadotrophe ceases. Meyer and Wolfe (191), showed that inhibitory effects, and the precipitins, in the blood of monkeys receiving crude and purified PMS preparations disappeared by 59 days after cessation of injection. In human beings the effects of the injection of PMS are reported, Jailer and Leathem (145), to disappear within four months.

#### G. SUMMARY

The antigenadotrophic substances in serum produced by chronic injection of a gonadotrophic agent from one species into another are usually not specific for the donor species. However, as has already been mentioned, pregnant women do not develop antibodies to human chorionic gonadotrophin nor does this hormone usually produce antigenadotrophic agents when injected into human beings (Spence, Scowen, and Rowlands, 256), and we have also mentioned that pregnant mares do not develop antigenadotrophic substances to equine gonadotrophins. Nor does the rat develop anti-gonadotrophins on chronic implantation of rat pituitaries (Katzman, Wade, and Doisy, 151). In experiments with parabiotic rats, the ovary of the female rat parabiotically united with a castrate rat continues to react for long periods to the increased amounts of hormone produced by the castrate pituitary. There is a tendency for less antigenadotrophin formation from the injection of equine gonadotrophin into heterologous species as the hormone is more purified (Leathem and

Abarbanel, 165). McShan, Wolfe, and Meyer (189), have also stressed the purity of the source material in antibody production. The antiserum produced in rabbits immunized against pure hog pituitary ICSH—detectable by precipitation reaction and reacting with 1 mg of ICSH protein—reacted only with swine ICSH, not with sheep or beef ICSH. This too could possibly be explained on the basis of species specific body proteins contaminating the ICSH in traces not detectable by physicochemical methods for the determination of purity.

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## CHAPTER VII

### The Hypophysis and Diabetes Mellitus

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#### I. Introduction

The fact that a deficiency of insulin produces the metabolic disturbances of diabetes mellitus is one of the elementary facts of physiology that is learned by every medical student no later than his first year of training. However, there is ample evidence that the control of the normal pattern of carbohydrate metabolism rests not in the mere presence or absence of insulin, but rather in a balance of insulin and a hypophyseal hormone or hormones which, in a sense, oppose or antagonize the physiological effects of insulin. The four observations which offer the strongest support for the concept that a balance of hormones is involved and that the balance is upset in the metabolic disturbance of diabetes mellitus are: first, the marked hypersensitivity to insulin that develops in hypophysectomized animals and that cannot be accounted for by secondary hypofunction of the other endocrine glands; second, the inability of hypophysectomized animals to maintain normal carbohydrate stores during fasting; third, the lessening

of the severity of all manifestations of diabetes mellitus which follows hypophysectomy of depancreatized animals and the ability of such doubly operated animals to survive for long periods of time without insulin; and fourth, the production of temporary or permanent diabetes mellitus in normal animals with crude hypophyseal extracts and reinvocation of severe diabetes mellitus in hypophysectomized-depancreatized animals by such extracts. There is evidence that the hypophyseal control of carbohydrate metabolism is at least dual, being in part independent of the adrenal cortex and in part mediated by trophic control of the adrenal cortex via the adrenocorticotropic hormone (ACTH).

It is apparent that the major questions arising from the above types of observations are concerned with the mechanisms by which the effects are produced and the identity of the hypophyseal hormones whose deficiency or excess are responsible for the phenomena following hypophysectomy or the administration of crude anterior-lobe extracts. The recent isolation in chemically and biologically pure form of hypophyseal growth hormone (GH) and ACTH (55,56,84,87) has made possible some new observations regarding the identity of the hypophyseal hormones involved, as pure hormones are now available for biological work. It is the purpose of the present review to present the major experimental data relating to the four points outlined above and to summarize the more recent work carried out with pure hormones. No exhaustive review of the literature or older work is to be attempted. For excellent reviews and bibliographies, the reader is referred to such papers as those by Russell (76), Houssay (41-43), Long (57,58), or Young (92).

## II. Hypersensitivity to Insulin after Hypophysectomy

The hypersensitivity to insulin of hypophysectomized dogs was first pointed out by Houssay and Magenta in 1924 and subsequently has been confirmed by many observers in various species of animals. Similar hypersensitivity to insulin is a well recognized clinical phenomenon in Simmond's disease. This hypersensitivity is due to the removal of the anterior lobe rather than the posterior lobe (51,53,71) and cannot entirely be accounted for as due to functional subnormality of adrenal cortex, adrenal medulla, or thyroid. Hypothalamic injury in dogs (20) produces a similar hypersensitivity to insulin. Unless one assumes hypothalamic control of the hypophysis, the hypothalamic injury possibly associated with hypophysectomy cannot, by itself, entirely account for the hypersensitivity to insulin, since *anterior-hypophyseal extracts restore the insulin sensitivity to normal.*

The blood-glucose-lowering effect of insulin is due to the fact that insulin

accelerates the shift of glucose from blood to peripheral tissues where it is oxidized and/or stored. As hypoglycemia is produced there is an increased hepatic output of glucose. Hypersensitivity to insulin could be due either to an increased effectiveness of insulin in accelerating the shift of glucose from blood to peripheral tissues or (and) to a decrease in the compensatory hepatic glucose output. There is evidence that both factors may be involved in the hypersensitivity following hypophysectomy. Eviscerated hypophysectomized rats are more sensitive to insulin than are eviscerated normal rats (11). This indicates that in this species part of the hypersensitivity to insulin is of extrahepatic origin. Further evidence to support this view is found in the observation (40) that anti-insulin effects are demonstrable in hepatectomized rabbits and in eviscerated rats (11). That there is a hepatic component to the insulin hypersensitivity of hypophysectomized dogs was demonstrated quite conclusively by Crandall and Cherry (24), who showed in angiostomized dogs that during insulin hypoglycemia there was a reduced hepatic glucose output in hypophysectomized dogs as compared to normal controls.

Since the increased hepatic glucose output during hypoglycemia is due in part, at least, to the release of adrenaline from the adrenal medulla, the question arises as to whether this mechanism is at fault following hypophysectomy. There is no good evidence that there are any functional abnormalities of the adrenal medulla following hypophysectomy, but there is good evidence that hepatic glycogen is not normally mobilized by adrenaline in hypophysectomized dogs (25). On the other hand there seems to be no similar impairment in hypophysectomized rats (82). Further clarification of these points must await further work.

That crude anterior-lobe extract would correct the hypersensitivity to insulin of hypophysectomized toads was demonstrated in 1929 (51) and the observation was extended to the dog in 1932 (26) and to rabbits in 1934 (23). The name glycotrophic has been suggested for the component of anterior-lobe extracts which exerts this effect (Young, 89) and Young has presented evidence that it is not identical with the lactogenic, thyrotrophic, or gonadotrophic hormones. ACTH, acting through the adrenal cortex, has anti-insulin effects, as will be discussed subsequently, but it is doubtful that the anti-insulin activity of crude extracts is due entirely to their ACTH content. Evidence for a partial nonhepatic origin of anti-insulin effects has been presented above and it is interesting to speculate that the apparent antagonism between insulin and crude anterior-pituitary extracts on the hexokinase system (72) may be involved in this.

The *in vitro* inhibition of hexokinase by crude anterior pituitary extracts and the removal of the inhibition by insulin offers a reasonable ex-

planation of *in vivo* anti-insulin effects of such extracts. However, this phenomenon does not adequately explain the hypersensitivity to insulin of hypophysectomized animals, since there is no anterior hypophyseal factor present in these animals and insulin alone apparently does not alter hexokinase activity. The *in vitro* inhibition of hexokinase is not produced by pure GH, lactogenic hormone, or ACTH (20a). The identity of this hexokinase inhibiting factor is obscure. It has been reported that there is no relation between the diabetogenic potency of crude hypophyseal extracts *in vivo* and the hexokinase inhibiting activity of the extracts *in vitro* (72a). Other workers (15a, 85a) have questioned whether hypophyseal inhibition of hexokinase is a contributing factor to the altered metabolism of pancreatic diabetes.

### III. Carbohydrate Stores of Hypophysectomized Animals

When hypophysectomized animals are fully fed a normal diet they have normal levels of blood glucose, and liver and muscle glycogen. However, if they are fasted they develop hypoglycemia and abnormally low liver and muscle glycogen stores. This has been demonstrated in the toad (47), in the rabbit (22), in the dog (41), and in the rat (75). In hypophysectomized rats this increased rate of disappearance of carbohydrate stores during fasting is associated with an R.Q. that is higher than normal. This may be taken as evidence that carbohydrate is being oxidized at an abnormally rapid rate (32). If a crude anterior-pituitary extract is administered only during the fasting period the R.Q. is depressed to normal levels and the muscle glycogen is maintained at normal levels (77). This effect is obtained in hypophysectomized-adrenalectomized animals and thus is *not mediated via ACTH* (81). Although under appropriate conditions cortical extract may act synergistically with anterior pituitary extracts (79), amounts of cortical extract which will maintain normal muscle glycogen in adrenalectomized rats are ineffective in hypophysectomized rats (4). If after a fast glucose is fed to hypophysectomized rats the R.Q. rises higher than that of normal rats, and less than a normal amount of the absorbed glucose is recoverable as glycogen or as glucose in the body fluids (77). This again supports the concept that carbohydrate oxidation is proceeding at an abnormally rapid rate in hypophysectomized rats.

One must consider the possibility that the increased rate of disappearance of carbohydrate stores during fasting of hypophysectomized rats and the decreased recovery of absorbed glucose as glycogen could be due to decreased gluconeogenesis rather than to increased glucose oxidation. However, the difference in urinary nitrogen excretion between fasting normal and hypophysectomized rats is insufficient to enable one to explain the loss

of carbohydrate on the basis of faulty gluconeogenesis from protein. Thus, if decreased gluconeogenesis is the factor, it must be gluconeogenesis from fat. This would imply that gluconeogenesis from fat occurs in normal rats in the presence of a large exogenous supply of carbohydrate. This seems unlikely. The carbohydrate stores of fed hypophysectomized rats maintained on a carbohydrate-free diet are normal (unpublished observation). This furnishes strong evidence that there is no major fault in gluconeogenesis in these animals.

Much better evidence that there is an increased oxidation of glucose after hypophysectomy is furnished by the observation of Greeley (37,38) and Russell (80) that the rate at which glucose must be administered by infusion to eviscerated rabbits and rats to maintain normal blood glucose levels is increased in hypophysectomized animals. Anterior pituitary extracts reduce the glucose requirement and maintain normal muscle glycogen. Cortical extracts may reduce the glucose requirement but do not maintain muscle glycogen. In these experiments gluconeogenesis can hardly be involved and we are dealing directly with extrahepatic influences upon glucose utilization.

In dogs the evidence for an increased rate of glucose utilization after hypophysectomy is much less convincing. Hypophysectomized dogs will maintain a normal blood glucose while on a high-carbohydrate or high-protein diet, but develop hypoglycemia while on a high-fat diet (85). Eviscerated hypophysectomized dogs do not have an increased requirement for infused glucose to maintain normal blood glucose levels (48). As pointed out above, hypophysectomized dogs exhibit definite abnormalities in hepatic glucose output and in hepatic glycogen mobilization. In addition hypophysectomized dogs during fasting excrete much less nitrogen than control animals (15). It would seem as though following hypophysectomy there may be both an increased peripheral utilization of carbohydrates and a reduction of gluconeogenesis (particularly during fasting). In fasting rats the former predominates while in fasting dogs the latter predominates. However, observations in hypophysectomized-depancreatized dogs suggest that the anterior pituitary is concerned with the utilization of carbohydrate when exogenous carbohydrate is supplied to this species as well.

#### IV. Hypophysectomy of Depancreatized Animals

The fundamental discovery of Houssay that hypophysectomy lessens the severity of all manifestations of diabetes mellitus has been abundantly confirmed in mammals, fish, amphibians, and reptiles. Documentation of these observations seems superfluous for this review and for bibliography

the reader is referred to the review articles. Following hypophysectomy of depancreatized dogs, there is reduction in hyperglycemia, hyperlipemia, ketonuria, glycosuria, and urinary nitrogen. Such doubly operated dogs may survive from 6-9 months without exogenous insulin, whereas depancreatized dogs without insulin seldom survive more than three weeks. They have greater resistance to infection and their wounds heal better than those of depancreatized dogs. The doubly operated animals are hypersensitive to insulin and develop hypoglycemia upon fasting. However, the animals cannot be regarded as having had their diabetes cured, since they still exhibit hyperglycemia and glycosuria if fed carbohydrate and have a diabetic type of glucose tolerance curve. When glucose is administered the R.Q. rises and not all of the glucose is excreted in the urine. Certainly the ability to utilize exogenous carbohydrate has been improved. The above effects are due to the removal of the anterior rather than the posterior lobe. Adrenalectomy (not removal of the adrenal medulla) produces an attenuation of pancreatic diabetes that is quite similar to that following hypophysectomy; gonadectomy or thyroidectomy does not.

All of the above facts demonstrate quite clearly that the presence of the anterior hypophysis is a factor which aggravates the disturbances due to insulin deficiency. Diabetes mellitus is a metabolic disease of endocrine equilibrium involving the secretion of insulin on the one hand, and the secretions of the hypophysis and the adrenal cortex on the other. The question of the identity of the hypophyseal factor or factors which are concerned in this equilibrium can only be clarified by a consideration of the effectiveness of crude and purified hypophyseal preparations in the reinvocation of severe diabetes in such animals or in the production of diabetes in normal animals.

## V. Enhancement or Production of Diabetes Mellitus by Hypophyseal Extracts

### A. EFFECTS IN HYPOPHYSECTOMIZED-DEPANCREATIZED ANIMALS

The administration of suitable crude hypophyseal extract to an hypophysectomized-depancreatized animal reinvokes typical, severe diabetes mellitus with marked hyperglycemia, glycosuria, ketonuria, acidosis, and death in diabetic coma. This was first pointed out by Houssay and has been adequately confirmed. For details and bibliography the reader is referred to the reviews. Two important deductions regarding the mechanism of action of hypophyseal extract can be made from these experiments. First, the diabetes-enhancing effect of the hypophyseal extract does not involve insulin antagonism, since it occurs in depancreatized animals that are receiving no exogenous insulin. As a corollary, it should be pointed out

that insulin action in the hypophysectomized animal does not involve the inhibition of hypophyseal hormones, since none are present. Second, the diabetes-enhancing effect of hypophyseal extracts is extra-pancreatic and cannot be due to suppression of insulin production, since no islets of Langerhans are present.

The enhancement of diabetes under these conditions plus the production of diabetes mellitus in normal animals has given rise to the concept that the anterior pituitary contains or secretes a diabetogenic hormone. As Professor Houssay has so well pointed out, this is a poor term. One should not think of the hypophysis as secreting a hormone whose physiological function is to produce diabetes mellitus. Rather one should think of the hypophysis as secreting hormones concerned directly or indirectly with the control of the metabolism of carbohydrate. When these are present in excess, relative to the available insulin, the metabolic abnormalities of diabetes mellitus are produced.

### B. PRODUCTION OF DIABETES MELLITUS IN NORMAL ANIMALS

Almost simultaneously in 1932 Evans, Myer, Simpson, and Reichert; Houssay, Biasotti, and Rietti; and Baumann and Marine reported that diabetes mellitus could be produced in normal dogs or rabbits by the injection of crude anterior-pituitary extracts. This diabetes may be either transient, occurring only during the injection of the extract, or permanent, persisting after cessation of injection of the extract. Houssay has suggested that the diabetes present during the period of injection of the extract be called hypophyseal diabetes and that the diabetes persisting after cessation of injection be called metahypophyseal diabetes. This terminology will be adhered to in this chapter. These observations have been thoroughly confirmed and documented in Houssay's laboratory and by others (18, 27-29, 88-91). Only large doses of crude extracts prepared from anterior lobes frozen almost immediately after removal at the abattoir have been effective in the production of diabetes in normal cats and dogs. Smaller doses are required in animals with a pancreas reduced in size by previous surgery. Hypophyseal diabetes can be produced in the absence of the thyroid, ovary, testis, adrenal medulla, adrenal cortex (see next section), or after splanchnic-nerve section. It is most easily produced in animals on a high-carbohydrate diet and is not observed if animals are fasted.

In the production of hypophyseal diabetes in the dog there usually is a latent period of from 1-3 days before hyperglycemia is produced. If the dose of crude extract is increased, the hyperglycemia can usually be maintained or intensified. It is associated with all the classical signs of diabetes mellitus: glycosuria, ketonuria, polyuria, polydipsia, hyperketonemia, hyperlipemia, diabetic glucose tolerance curves, weight loss, and increased

urinary nitrogen. During the period of injection the dogs show insulin resistance. In certain animals if the injections are prolonged the diabetes persists after the cessation of injection and goes into the phase of metahypophyseal diabetes. This likewise is more readily produced in animals with a pancreas reduced in size by previous surgery. An animal with metahypophyseal diabetes shows all the classical signs of diabetes which may be intensified with the passage of time. Such an animal is not insulin-resistant but responds as does the depancreatized animal to exogenous insulin, although it may survive for prolonged periods of time without exogenous insulin. Extirpation of the liver is followed by a rapid fall of blood glucose to hypoglycemic levels similar to that occurring in depancreatized animals. There is no reason for believing that the utilization of exogenous glucose differs qualitatively in such animals as compared to depancreatized animals. Perhaps it should be pointed out here that there are no reports of metahypophyseal diabetes following administration of pure hypophyseal hormones.

Finally, it should be pointed out that it is difficult to produce hypophyseal diabetes in normal rats and that metahypophyseal diabetes has not been reported in this species. However, in normal fasted rats and rabbits it is not difficult to produce metabolic aberrations which resemble those of insulin deficiency. The two most important of these effects are the production of increased ketonuria and hyperketonemia (16,65) and fatty livers (12,17). The former of these will be discussed in more detail in the section on effects of purified GH and ACTH.

### C. ROLE OF ADRENALS

As pointed out previously adrenalectomy produces an amelioration of pancreatic diabetes that is quite like that following hypophsectomy (61). This leaves but little doubt that the effects of hypophsectomy in pancreatic diabetes are in part due to secondary functional subnormality of the adrenal cortex, but it does not prove that such functional subnormality is the entire explanation of the amelioration of the diabetes. Crude ACTH enhances the diabetes of hypophsectomized, partially depancreatized animals only when their adrenal cortices are present (57,58). This is strong support for the view that part of the diabetogenic effects of crude extracts is due to their ACTH content and is mediated via the adrenal cortex. That the adrenocortical "11-oxygenated steroids" have similar effects is in perfect accord with this.

On the other hand there is ample evidence that crude anterior-pituitary extracts contain some factor (or factors) which operate independently of the adrenal cortex to intensify or to produce diabetes mellitus. Crude anterior-pituitary extracts produce or intensify diabetes in adrenalectomized

toads and dogs either with or without an intact pituitary (41,44,45). In diabetic adrenalectomized rats maintained upon constant doses of adrenocortical extracts the administration of hypophyseal extracts produces a further enhancement of their glycosuria (60). These facts are in accord with the direct inhibition of the hexokinase system which crude hypophyseal extracts have been reported to show (72), with the apparent synergism between hypophyseal extracts and cortical extracts in suppressing carbohydrate utilization (79,80), and with the maintenance of muscle glycogen in hypophysectomized, adrenalectomized rats by crude hypophyseal extracts (10). The identity of this factor operating independently of the adrenal is not entirely clear but evidence from recent experiments of another sort renders it highly probable that GH is operating here (23a, 69a).

#### D. ROLE OF PANCREAS

The histology of the islets of Langerhans and the insulin content of the pancreas during hypophyseal and metahypophyseal diabetes have been studied by conventional techniques in various laboratories (13, 14, 19, 62-64, 74). The ability of the pancreas to secrete insulin has been studied extensively by Houssay and collaborators (43, 49). These latter investigations were carried out in the following ingenious fashion. The pancreas of a dog with hypophyseal or metahypophyseal diabetes was transplanted into the neck of a depancreatized dog under chloralose anesthesia by means of vascular anastomoses between the carotid artery and external jugular vein of the recipient and the pancreaticoduodenal vessels of the donor. The recipient dog had hyperglycemia and the time required for the transplanted pancreas to correct this hyperglycemia was taken as measure of its ability to secrete insulin. The pancreas from a normal dog transplanted in this fashion will correct the hyperglycemia of the recipient diabetic dog in from 3-6 hours. If the pancreas from an experimental animal does not correct the hyperglycemia in 6 hours, reduced capacity to secrete insulin is indicated.

Evidence obtained from the above types of experiments show that after 2-3 days of hyperglycemia in hypophyseal diabetes there are hydropic changes in the beta cells of the islets of Langerhans, a reduced insulin content of the pancreas, and an impaired ability to secrete insulin. These abnormalities persist in metahypophyseal diabetes and there is gradual total destruction of beta cells. If hyperglycemia is prevented by diet, insulin, or phlorizin, the anatomical changes and the changes in insulin content are prevented. In the partially depancreatized cat with metahypophyseal diabetes there is a period of about 3 months in the early phase in which treatment with insulin, diet, or phlorizin will produce anatomical and func-

tional restoration of the islets (62-64). Apparently the production of normal blood glucose is the critical factor here.

As pointed out previously hypophyseal extracts have an extrapancreatic diabetes-enhancing effect which does not involve insulin antagonism. The fact that the pancreas of the hypophysectomized dog secretes insulin at a normal rate is further evidence against direct hypophyseal control of insulin production. However, the above facts concerning pancreatic effects in hypophyseal and metahypophyseal diabetes leave no doubt that the insulin-forming and -secreting mechanisms of the pancreas are injured under these circumstances. It is this injury that is responsible for the persistence of the metahypophyseal diabetes, essentially a pancreatic diabetes. How is this injury brought about? Is it a direct effect on the islets of Langerhans? Or is the effect of the pituitary extract to produce hyperglycemia by extrapancreatic mechanisms and does the hyperglycemia *per se* produce the islet changes? Or, are both hyperglycemia and a direct effect on the islets involved?

In view of the effectiveness of agents which maintain or restore a normal blood glucose in preventing or reversing the islet changes in hypophyseal and metahypophyseal diabetes, it would seem that hyperglycemia *per se*, rather than a direct effect of the extract, is the more important factor. The recent observation that intraperitoneal glucose injections will produce permanent diabetes in partially depancreatized cats seems to be in accord with this view (30). However, these animals had intact pituitaries and if some pituitary factor acts synergistically with hyperglycemia, the animals' own pituitaries could have been producing it. The production of hyperglycemia in normal dogs by glucose infusion does not impair the ability of the pancreas to secrete insulin (43,49), even though the duration and degree of the hyperglycemia are comparable to those following hypophyseal-extract injections which do impair the insulin-secreting ability of the pancreas. This lends support to the view that both hyperglycemia and a pituitary factor are operating in the production of islet injury. Perhaps, as Houssay has suggested, hypophyseal extract elevates the blood glucose by extrapancreatic mechanisms and the islet injury is produced by some hypophyseal factor which is effective only in the presence of hyperglycemia. It is apparent that at present no conclusive answers can be given to the questions raised above.

There is evidence which leads one to believe that the pancreas of the rat may behave differently than the pancreas of the dog. Extracts which in the dog produce or intensify diabetes in the rat produce increased rate of growth, islet hypertrophy, and increased insulin content (66,67,73). Indeed, nitrogen storage may precede the production of diabetes and the consequent nitrogen loss in cats and dogs and islet hyperplasia may precede

the exhaustive changes which accompany the onset of diabetes (74,93). An extract which will intensify diabetes in a depancreatized dog will promote nitrogen storage in the presence of adequate amounts of insulin (35). All crude extracts which have been successfully used in the production of hypophyseal and metahypophyseal diabetes have contained ACTH as well as GH. This GH content is undoubtedly responsible for the above-noted effects upon nitrogen storage. Are these effects brought about through an increased insulin production? Or, does GH injure the islets of Langerhans? To what extent does GH contribute to the diabetogenic effects of crude hypophyseal extracts? At least partial answers to these questions may be found in some of the recent work with pure GH which will be discussed later.

## VI. Effects of Adrenocorticotrophic Hormone and Growth Hormone

Relatively few metabolic studies relating to the phenomena previously discussed have been made with ACTH or GH preparations that fulfilled the electrophoretic, solubility, and biological criteria for purity. Some pertinent observations will be considered below.

### A. KETOGENIC EFFECTS

There is evidence that either or both hormones may, under appropriate experimental conditions, increase the degree of ketonemia and ketonuria of normal fasted rats and dogs or of diabetic rats. If normal male rats are fasted for 72 hours the administration of either hormone increases the urinary ketone bodies during the last 48 hours of the fast (7). The urinary nitrogen of the GH-treated animals is less than that of the controls, while the urinary nitrogen of the ACTH-treated animals is greater. That this increase in ketonuria is associated with an increase in ketonemia was demonstrated in acute experiments in which the hormones were administered at the end of a 24-hour fast. In these experiments a rise in blood ketone bodies was observed in the treated animals 3 hours later (Table I). The rate of disappearance of the blood ketone bodies following evisceration and nephrectomy is the same in the controls and in the ACTH- and GH-treated animals, suggesting that the increased ketonemia is due to increased production rather than reduced peripheral utilization. Single injections of ACTH have been shown to produce a transient increase in ketonemia in a normal dog (6). In severely diabetic rats which are maintained on a carbohydrate-free diet either hormone increases the degree of ketonuria.

One can only speculate regarding the mechanism of the ketogenic effects of these two hormones. The effects are produced only under conditions in which the endogenous carbohydrate stores are reduced to a minimum or exogenous carbohydrate is lacking. It seems reasonable to regard the keto-

genic effects as one manifestation of the altered metabolism produced by the hormones rather than as a primary effect. The fact that ACTH has this effect is not surprising in view of the demonstration that Compound E increases the ketonuria of patients with Addison's disease and diabetes mellitus (54,86). Perhaps the primary effect of GH is to reduce protein catabolism (as reflected by the reduced urinary nitrogen) and thus force the organism to derive a larger fraction of its total caloric requirement from fat.

### B. INSULIN RESISTANCE

A highly purified ACTH preparation has been reported to increase the insulin resistance of normal mice (36). Unpublished observations by the

TABLE I  
EFFECT OF GH AND ACTH ON BLOOD KETONE BODIES OF FASTED NORMAL RATS

Injection	No. animals	Initial blood, mg. %	3-hr. blood mg. %	Change	P*
Controls, no inj.	12	7.9 ± 0.94 <sup>a</sup>	8.0 ± 1.06	+0.1 ± 0.85	
3 mg. albumin	5	6.8 ± 1.45	6.1 ± 1.54	-0.7 ± 0.18	0.55
0.5 mg. ACTH	7	7.8 ± 1.41	13.0 ± 2.18	+5.2 ± 1.83	0.01
1.5 mg. ACTH	6	6.9 ± 0.79	16.3 ± 2.52	+9.3 ± 2.25	0.01
3 mg. ACTH	9	7.9 ± 1.52	25.6 ± 1.97	+17.7 ± 2.65	0.01
0.5 mg. GH	8	6.4 ± 0.85	10.1 ± 1.25	+3.7 ± 0.57	0.01
1.5 mg. GH	7	7.0 ± 0.84	11.4 ± 1.32	+4.4 ± 0.80	0.01
3 mg. GH	8	7.4 ± 1.48	16.2 ± 2.00	+8.8 ± 1.70	0.01

\* Standard deviation of the mean.

<sup>b</sup> From Fisher's table of t. A value of 0.05 or less is considered significant.

authors suggest that electrophoretically and biologically pure ACTH produces some increase in the insulin resistance of normal rats but critical evidence on this score is lacking. However, from experiments with diabetic rats receiving exogenous insulin and then given ACTH there is strong evidence that it may be considered to have anti-insulin effects (9). In such experiments ACTH consistently increases both the glycosuria and urinary nitrogen, *i.e.*, negates the insulin effect. When, in addition, one considers that it produces hyperglycemia, glycosuria, and diabetic glucose tolerance curves in normal men and rats on a high-carbohydrate diet (21,52), there seems but little doubt that the ACTH content of crude anterior-hypophyseal extracts contributes significantly to their anti-insulin effects.

With regard to the question of anti-insulin effects of GH in normal animals our own observations have suggested some insulin resistance but have not been entirely convincing. Recently, although no details were

given it has been reported (69a) that pretreatment with pure GH reduces the hypoglycemic effect of insulin in normal as well as diabetic rats. However, in diabetic rats maintained on a constant food intake and given insulin, GH consistently produces a further reduction of urinary nitrogen (9). GH certainly is not anti-insulin with respect to its effect upon nitrogen metabolism. On the other hand, it occasionally increases the glycosuria of such animals and so in this sense it may be regarded as having minimal anti-insulin effects. Obviously, much further work remains to be done in this field.

#### C. CARBOHYDRATE STORES OF HYPOPHYSECTOMIZED RATS

As pointed out in an earlier section, the administration of a suitable crude anterior-hypophyseal extract to an hypophysectomized rat during the period of a 24-hour fast will result in the maintenance of normal muscle glycogen stores even though the blood glucose and liver glycogen are at sub-normal levels. This effect of crude extract is demonstrable in hypophysectomized-adrenalectomized rats and thus is independent of the adrenal cortex. On the other hand, if hypophysectomized rats are treated with ACTH so as to produce hypertrophy of their adrenals, are allowed free access to food during this period, and then are fasted, their blood glucose and liver and muscle glycogen levels are above those of controls (3,39). Treatment with Compound B has a similar effect (59,83). However, if ACTH is administered to hypophysectomized rats only during the period of the 24-hour fast, the level of muscle glycogen is not maintained (10). Thus it seems clear that there are two anterior-hypophyseal factors concerned with the maintenance of carbohydrate stores in fasting hypophysectomized rats: first, ACTH, which stimulates the adrenal cortex to release steroids having biological activity like Compound B or E and which are responsible for the effect under appropriate conditions; second, a factor which operates independently of the adrenal cortex and does not require the presence of adrenocortical steroids and which presumably has its effect because it suppresses the peripheral oxidation of carbohydrate.

The identity of this latter factor is not clear and whether it is GH remains an open question. Electrophoretically homogeneous GH (87) has this effect. Other highly purified GH preparations have exhibited this effect (39, 78), but increasing GH potency has been associated with decreasing potency per unit GH activity with regard to the ability of such preparations to maintain muscle glycogen.

#### D. EFFECTS ON PANCREAS

It has been reported that highly purified GH decreases the insulin content of the rat pancreas (34) and that pure GH inhibits the increased insulin production which hyperglycemia produces in the perfused rat pancreas (1).

The latter seems inconsistent with the observed hypoglycemic effects of GH in fasted normal rats (69a) and with the effects of GH in hypophysectomized diabetic rats, which will be discussed later. There is no experimental evidence which directly relates either of these pure hormones to the changes in islet histology, pancreatic insulin content, or the ability of the pancreas to secrete insulin, which are encountered in the production of hypophyseal or metahypophyseal diabetes in dogs or cats.

#### E. PRODUCTION OF GLYCOSURIA IN NORMAL ANIMALS

ACTH has been reported to produce glycosuria in normal animals. In human subjects elevated fasting blood glucose levels and an increase in liver glycogen have been produced (33) as well as hyperglycemia, glycosuria, and diabetic glucose tolerance curves (21). In normal rats force-fed a high-carbohydrate diet the administration of 7 mg. ACTH/day produces hyperglycemia and glycosuria (52). It is also worthy of note that this hormone has been reported to increase the blood glucose levels of hypophysectomized toads (43). Save that the adrenal is necessary, the mechanism of this action of ACTH is not clear. It is certain that, if one uses the increase in urinary nitrogen excretion as a criterion of increased protein catabolism, the glucose in the urine cannot be accounted for as coming from the additional protein broken down. In all of these experiments there has been adequate dietary carbohydrate present. Presumably there is some interference with the oxidation, storage, or conversion to fat of the dietary carbohydrate. The alternative hypothesis would involve gluconeogenesis from fat in the face of a large dietary supply of carbohydrate which was largely being utilized.

Recently it has been shown that pure GH will produce glycosuria in intact adult cats (23a) at doses of 3 to 6 mg. per day. It would seem likely that in cats and dogs, GH is a more potent agent for the production of glycosuria than is ACTH, while in rats and man ACTH is the more potent diabetogenic agent. There is every reason to believe that both the GH and ACTH component of crude extracts contribute to the diabetogenic activity of such extracts.

#### F. EFFECTS IN DIABETIC ANIMALS

Biologically pure GH has been reported to increase the glycosuria of partially depancreatized rats allowed free access to sucrose in their drinking water (68). GH consistently produces a positive nitrogen balance in diabetic rats maintained on a constant food intake, either with or without exogenous insulin, and only occasionally increases the glycosuria of such animals (9). These results are inconsistent with the hypothesis that the

increased nitrogen storage which GH produces is secondary to an increased insulin production (70). If the reduction in urinary nitrogen were secondary to an increased insulin production, one would expect the glycosuria to decrease as well (as it does when diabetic animals are given insulin). Since these experiments were carried out in animals made diabetic by the administration of alloxan and which had survived for prolonged periods of time

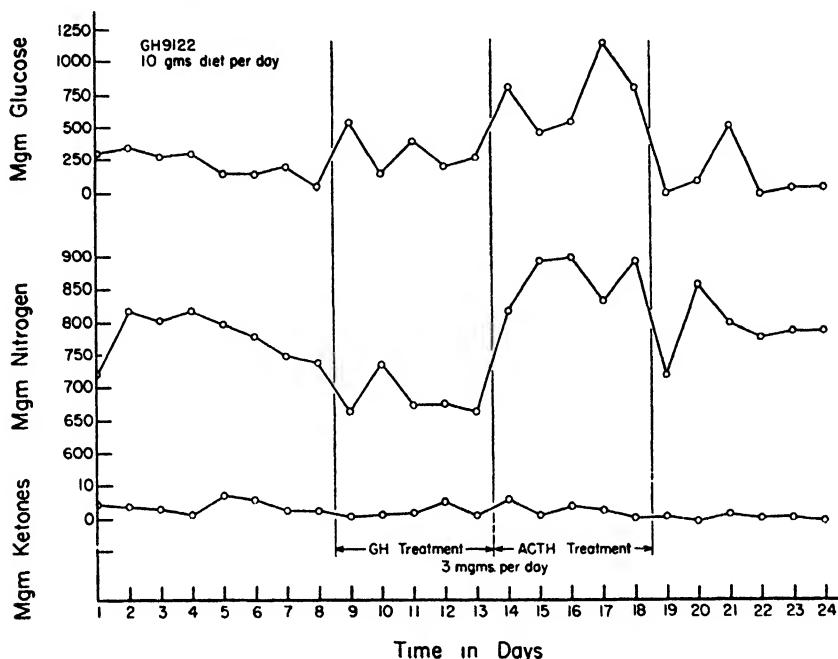


FIG. 1. Effect of GH and ACTH on urinary glucose, nitrogen, and ketone bodies of a diabetic rat maintained on a carbohydrate-free diet. Note minimal glycosuria in preinjection control period and slight, although not statistically significant, increase in glycosuria following administration of GH. ACTH produced, at the peak, a more than fourfold increase in glycosuria. GH reduced urinary nitrogen ACTH increased it. Neither hormone changed ketonuria level. Dietary intake constant throughout.

without exogenous insulin, presumably the rats had some residual functioning beta cells in their islets. Hence, these experiments offer no evidence relating to the question of the essentiality of insulin for the protein-anabolic effect of GH.

The experiments previously cited indicating that GH inhibits the increased insulin secretion produced by hyperglycemia in the perfused rat pancreas (1) are consistent with the occasional increase in glycosuria of diabetic animals noted above. However, if this inhibition of insulin secre-

tion occurs in the diabetic rat, the primary effect of GH on protein anabolism predominates over any effects due to a further reduction of insulin secretion, since nitrogen storage is produced.

When GH was administered to diabetic rats maintained on a carbohydrate-free diet, in each of six experiments, there was a slight increase in the average glycosuria, but in no case was the increase statistically significant (8). In each experiment there was significant nitrogen storage. The

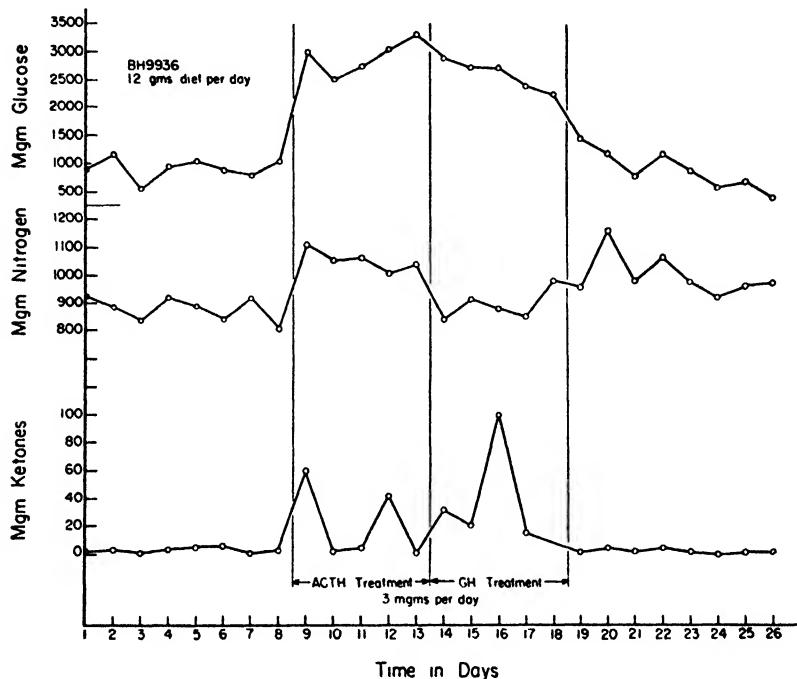


FIG. 2. Effect of GH and ACTH on urinary glucose, nitrogen, and ketone bodies of a diabetic rat maintained on a carbohydrate-free diet. Note more marked glycosuria during control period than shown in Fig. 1. Note striking increase in glycosuria, ketonuria, and urinary nitrogen during ACTH administration. Glycosuria declined during GH administration, but ketonuria was enhanced.

same considerations regarding insulin secretion as were discussed above pertain to these experiments. When the glycosuria was severe, GH also increased the ketonuria of these animals (Figs. 1 and 2).

When GH was administered to a group of hypophysectomized diabetic rats, again nitrogen storage was produced, but in these experiments there was no increase in glycosuria (5) (Fig. 3). This seems inconsistent with the previous results, as one would expect such a "Houssay" rat to be the most sensitive test animal for any diabetes-enhancing effect of GH. Is it possible

that hypophysectomy alters the pancreatic response to GH? It is possible that if one mechanism of GH action is to *suppress the hyperglycemia-induced secretion of insulin*, GH would have no effect in hypophysectomized diabetic rats, since they are essentially aglycosuric and hence have no hyperglycemia of any degree. Clarification of these questions must await further

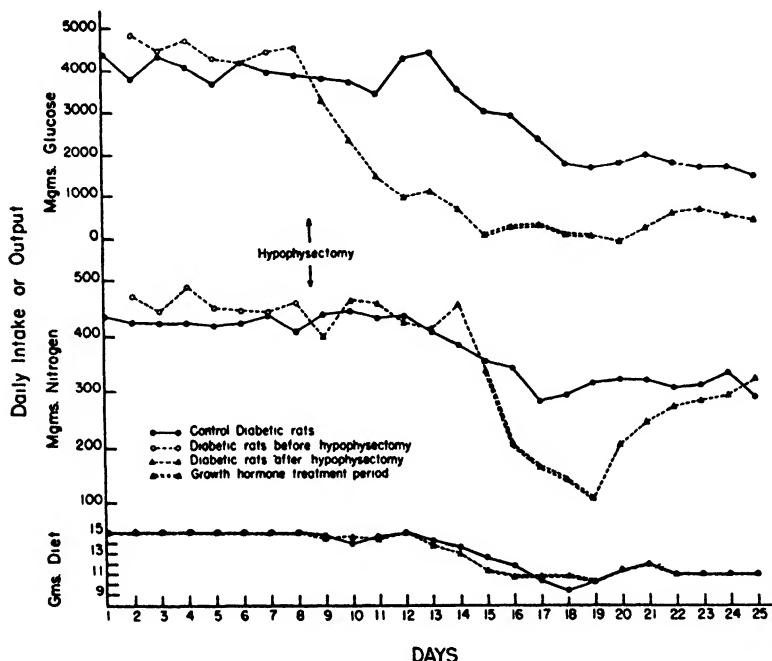


FIG. 3. Daily food consumption, urinary nitrogen, and urinary glucose of hypophysectomized diabetic rats treated with GH. Each curve represents average of four or five animals. Hypophysectomized diabetic rats pair-fed with the control diabetic rats. Diet contained about 60% carbohydrate. Note striking reduction in glycosuria following hypophysectomy and not accounted for by reduction in food intake. Note lack of effect of GH on glycosuria and marked nitrogen storage produced by GH.

experimental work, as must the question as to whether GH influences the secretion of a hyperglycemic principle by the pancreas.

In our laboratory the administration of ACTH to diabetic rats maintained on a constant food intake resulted in an increase in glycosuria as well as urinary nitrogen regardless of whether exogenous insulin was given (9). As in the production of glycosuria in normal animals the additional glucose in the urine could not be accounted for as having come from the additional protein catabolized under the influence of the hormone. In diabetic rats maintained on a carbohydrate-free diet, in which all glucose in the urine

must have been derived from noncarbohydrate precursors, ACTH likewise produced a rise in both glycosuria and urinary nitrogen. In this case the glucose-nitrogen ratios rose also, although they never exceeded 3.65 (8). In animals with marked glycosuria and high glucose-nitrogen ratios an increase in ketonuria was also produced. The most striking increases in glycosuria occurred following the administration of this hormone to hypophsectomized diabetic rats (5) (Fig. 4). In these animals also there was

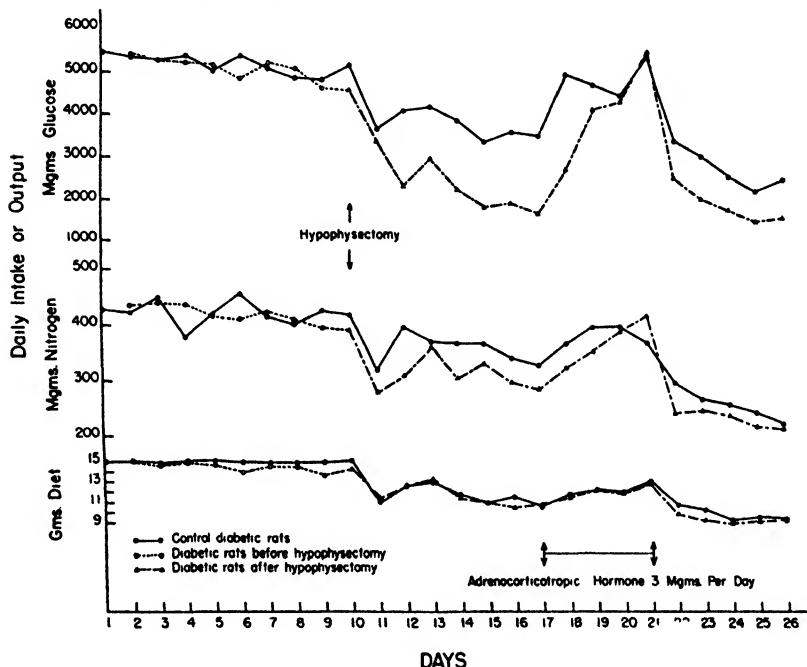


FIG. 4. Daily food consumption, urinary nitrogen, and urinary glucose of hypophsectomized diabetic rats treated with ACTH. Each curve represents average of four or five animals; the two groups pair-fed same diet as in Fig. 3. Note striking increase in glycosuria during period of ACTH treatment and prompt decrease in glycosuria following cessation of treatment. Note urinary nitrogen increase and that gluconeogenesis from protein cannot account for the glycosuria.

the greatest discrepancy between the increase in glycosuria and the increase in protein catabolism.

In all the above experiments both hormones were administered at the same dose level, *i.e.*, usually 3 mg./day/animal. Other dose levels or combinations of the hormones have not as yet been tried. It seems clear that, at these doses and under these conditions, ACTH is a potent diabetes-enhancing agent. GH, on the other hand, has effects like insulin upon protein metabolism in the diabetic rat while under appropriate conditions it

produces a striking enhancement of the ketonuria. Its effect upon glycosuria is minimal. As mentioned earlier there is good reason to believe that the diabetes-enhancing effects of crude anterior-pituitary extracts cannot be accounted for solely by their ACTH content. Whether this additional component of crude extract is the GH alone or GH plus some additional factor is a question to which a final answer cannot yet be given.

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## CHAPTER VIII

### Hormones of the Posterior Pituitary

BY H. WARING AND F. W. LANDGREBE

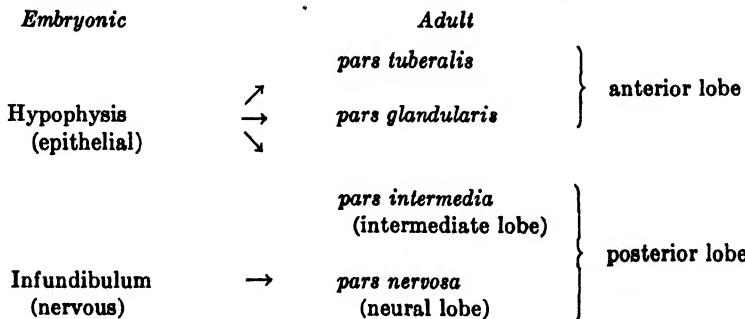
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## I. Introduction

The pituitary gland has a double embryonic origin: an epithelial up-growth (hypophysis) from the stomodeal region unites with a downgrowth from the third ventricle of the brain (infundibulum). The adult derivatives from these two rudiments are shown in the following scheme:



Fresh or acetone-dried pituitary glands of most mammals, including ox, are readily divided at the hypophyseal cleft into what are customarily called

the anterior and posterior lobes. The latter contains both neural and epithelial tissue and is more correctly designated the neurointermediate lobe.

Appraisal of the endocrine status of any tissue requires, *inter alia*, information regarding dysfunction evoked by its removal as well as information on the effect of injecting extracts (100). For this reason we have adopted the convention of classifying biological information into two sections: (a) "pharmacology," which includes information about injections alone and (b) "physiology," a category which is reserved for integrated information from several sources such as extirpation, injection, and excitant content of blood.

Extirpation experiments involving the posterior lobe are especially difficult to interpret for two reasons: surgical removal of the posterior lobe *sensu stricto* without damage to the anterior lobe is difficult, and removal of the lobe inevitably severs nerve tracts to the hypothalamic region. The latter is particularly embarrassing because of the doubt which still exists regarding the exact limitation of endocrine function between the posterior lobe and its associated hypothalamic elements (153). Consequently it is not surprising that we have more unequivocal information about the effect of injections than of extirpations.

Most workers who have studied the effects of posterior-lobe extracts used ox material, so their extracts contain excitants from both neural and epithelial tissue. At the present time four effects of unfractionated posterior-lobe extract are universally acknowledged to be evoked by posterior-lobe excitants: (1) increase of blood pressure after intravenous injection into anesthetized or spinal animals, (2) contraction of the mammalian uterus, (3) antidiuresis in mammals, and (4) melanophore expansion in lower vertebrates. Many other results of injection of extracts have, however, been observed, including other effects on the circulation, effects on smooth muscle such as stomach and bowel, effects on lymph and edema fluid formation, and effects on carbohydrate, salt, and water metabolism. It cannot at present be claimed with confidence that *all* these effects are due to posterior-lobe excitants themselves, because some workers have used preparations which contain a variety of possible impurities, some of which are themselves pharmacologically active. Those which are well substantiated are detailed later.

Of the four effects of posterior-lobe extracts mentioned above, the first three are attributable to neural-lobe tissue and have been termed pressor, oxytocic, and antidiuretic effects, respectively. These terms have been derived from the effects observed on animals in the biological methods used for the assay of extracts.

Information from several sources (*vide infra*) shows that for the present there is no reason to believe that autacoids from the *pars intermedia* and

*pars nervosa* have any functional relationship in the intact animal, nor do extracts from the two influence one another *directly* in experiments at the pharmacological level. Consequently in the present article we shall consider them separately.

## II. Seat of Origin of Posterior-Lobe Properties

Whole extracts of posterior (neurointermediate) lobe evoke a variety of responses and we have to inquire whether the excitants are manufactured in the intermediate or neural portion of the gland. There are three kinds of evidence that leave no doubt that the melanophore-expanding ("B") hormone is produced by the intermediate lobe: (a) in species in which a discrete *pars intermedia* is lacking (cetaceans, armadillo, chicken, and manatee) "B" is present in the anterior and *not* in the neural lobe (65,118,155); in elasmobranchs, in which there is only a trace of neural tissue in the posterior lobe, there is abundant "B" (28); (b) posterior lobes from animals in which degeneration of the neural elements has been caused by hypothalamic lesions still contain normal amounts of "B" (39); (c) tissue cultures of the intermediate lobe alone have melanophore-expanding activity (4,64,72).

Reluctance of investigators to ascribe a secretory function to the type of cell shown by ordinary techniques in the *pars nervosa* early led to the view that all properties characteristic of posterior-lobe extracts were manufactured in the intermediate lobe. According to this view the admittedly large amounts detectable in the neural lobe would be stored only. A first step to throwing doubt on this was van Dyke's (205) observations. He separated the two lobes, controlling his experiment by microscopic examination of the separated tissues. Extracts of the *pars nervosa* exhibited no "B" activity and had a much higher titer of pressor, oxytocic, and anti-diuretic properties.

There is now convincing evidence that pressor, anti-diuretic, and oxytocic properties arise from the *pars nervosa*. This evidence can be epitomized as follows: (a) in species in which the *pars intermedia* is lacking, neural-lobe extracts have the usual properties; the posterior lobe of elasmobranchs has no detectable pressor or oxytocic properties<sup>1</sup>; (b) after section of the hypothalamic tract the usual properties are missing (39); (c) tissue cultures of neural lobe mixed with intermediate lobe have pressor and "B" activities (4,64,72).

Recent research has shown the presence of gland cells among the fibers of the *pars nervosa*. Although the evidence is not conclusive, these cells are

<sup>1</sup> In view of several claims to have detected oxytocic properties in elasmobranch pituitaries we have made exhaustive tests for both pressor (rat) and oxytocic (guinea pig uterus) properties in glands excised from living fish. We found neither. We are inclined to ascribe previous workers' findings to the presence of histamine.

probably the source of secretion. The active substance presumably comes from either the nervous tissue or these gland cells. If the former degenerates when severed from its central connection, the fact that tissue-cultured *pars nervosa* retains its pressor properties would imply that the gland cells are the source of activity. Various names have been applied to these gland cells and it is not at all certain that all workers are referring to the same cell. Bucy's term "pitucyte" is the name most commonly favored. Wang (218) was able to show that pitucytes from pituitaries "at rest" contained granules which are not present after vagal stimulation (Fig. 1). Since vagal

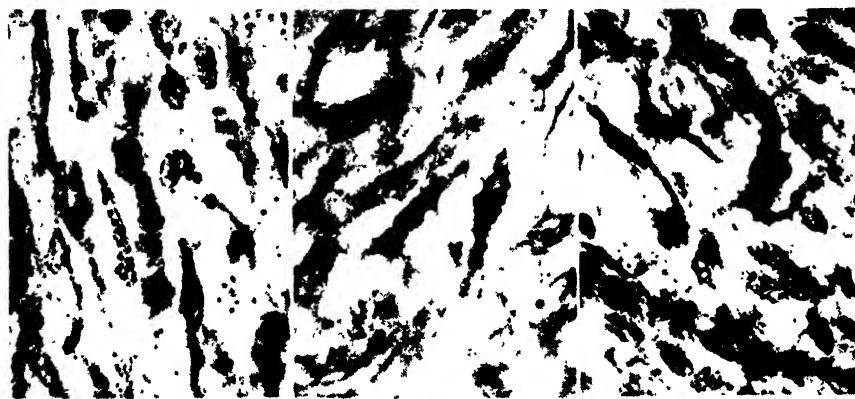


FIG. 1 Photomicrograph of the *pars nervosa* of the dog—Penfield's modification of del-Rio-Hortega silver carbonate stain,  $\times 516$ . *a*, Normal dog (7 8463, ♀, 8.1 kg.), showing pitucytes at resting condition. Note presence of granules in ectoplasm and long processes of cells. *b*, Dog (7 8462, ♀, 9.1 kg.) after afferent vagal stimulation. Note absence of granules in ectoplasm and loss of processes of cells. *c*, Dog (7 8475, ♀, 8.2 kg.) after stimulation of sympathetics. Note presence of granules in ectoplasm and long processes of cells (218).

stimulation reduces the quantity of excitant in the gland, it seems likely that these granules are secretion products. Using a different technique Gersh (67) described changes in fatty inclusions which he related to the known changes of excitant content after dehydration. Hickey *et al.* (98) were unable to confirm this.

The *pars nervosa*, pituitary stalk, and median eminence of the hypothalamus, which have the same embryological origin, share many features in common. Each contains a mass of nonmyelinated nerve fibers and Gersh has described glandular cells in the hypothalamus similar to those in the *pars nervosa*. This appears to provide the necessary cytological basis for the production of neural-lobe activities by the hypothalamus. Because of this, and data from physiological tests, we have in our account adopted

Fisher's term neurohypophysis to include collectively all the aforementioned parts.

Assuming the pituicytes to be the secretory elements, the function of the nerve fibers has to be considered. It seems to us that their presence is sufficiently explained by the evidence for a reflex connection between the vagus and supraopticohypophyseal tract (page 479) and evidence for the conduction of emotional excitement (page 481) to the neurohypophysis.

It seems to have been generally accepted, without evidence for or against, that "B" is released from the intermediate lobe directly into circulation. At one time there was support for the view that neural-lobe secretion passed directly into the spinal fluid (206). No trustworthy assays support this view, and it is now generally agreed that these secretions go directly into circulation; the neurohypophysis has a fairly rich blood supply with venous draining into the hypophyseal portal vessel.

### III. Neural Lobe

#### A. BIOLOGICAL ASSAY

Biological assay of an excitant is always necessary when it has not been isolated as a pure chemical; and even when the chemical nature is known biological assay is required for estimating quantities in circulation too small for chemical estimation. Two principles have been employed. Early workers defined a unit of activity in terms of a predetermined, arbitrarily specified response of the test animal, *e.g.*, a mouse unit of estrin. This method has now been abandoned because of the variations among different stocks of the same species and also in the same stock at different times. The method now generally adopted is to select a stable extract, preferably in the form of a dry powder, as a standard. International-Standard extracts are kept at a distribution center and an international unit (I.U.) is defined as the amount of activity in a certain weighed quantity of the extract (25). Biological assay then becomes a method for the comparison of an unknown extract to this standard. This system, used for the assay of posterior-lobe extracts, is operated in the belief, which appears to be justified, that the dried powder will retain its activity undiminished for many years. So long as the unknown is material similar to the standard, the results of an assay are largely independent of the actual details of technique. If the extract has been treated in any way, as in methods of fractionation, different techniques might yield different results when the unknown extract is measured against the standard (*e.g.*, p. 496). There is an International-Standard posterior-lobe powder and it has been agreed that one international unit of activity is the amount contained in 0.5 mg. when an extract is made from it as prescribed (127).

Three methods are in current use for the assay of neural-lobe activity (15). The first uses the rise of blood pressure produced by an extract after *intravenous* injection into a fully anesthetized or spinal animal as a test effect, and this property of the extract is called its pressor effect. The second method uses the contraction of the uterus of a virgin guinea pig *in vitro* as the test effect and this is called the oxytocic action of the extract. The third method exploits the antidiuretic action of the extract in intact unanesthetized animals.

These terms have led, chiefly by implication, to some confusion among clinicians and others. Pressor potency of an unknown is measured by comparing it to that of a standard extract of International Standard powder after *intravenous* injection into a fully anesthetized or spinal animal, *i.e.*, under very special conditions. The use of the term "pressor" is justified for the precise delimitation of a property as a necessary preliminary to its chemical isolation, but it provides no guide, as its name might imply, to the blood-pressure-raising potentialities of the extract as ordinarily employed in clinical practice, *viz.*, intramuscular injection into the unanesthetized subject.

This reservation is equally true for oxytocic, and to a lesser extent for antidiuretic, properties. Oxytocic activity is measured *in vitro* and the saline must be rigidly standardized to obtain comparable figures. If figures for oxytocic potency obtained under the special conditions mentioned have any meaning as a guide to what the extract will do under the entirely different conditions obtaining in clinical practice it is purely fortuitous; the only thing in common between the assay method using the guinea pig uterus *in vitro* and intramuscular injection into the human subject is the *name* of the effector organ to be activated. It is important to emphasize this point because many clinicians are misled into believing that an extract with high oxytocic titer will necessarily stimulate the human uterus *in vivo*. All we are justified in saying is that *under certain circumstances* the official oxytocic figure gives a clue to effectiveness of the extract on the human womb.

In the methods to be outlined, the accuracy for *routine assays* is usually about 20% and this meets the requirements of the British and U. S. pharmacopoeias. Greater accuracy for research purposes can be obtained by repetitive assays.

### 1. Assay of Pressor Activity

Several methods have been described for the assay of pressor activity, of which we may note three. Two of these are in current use; the third offers substantial advantages over both.

a. *Injection of Extracts into Anesthetized Dogs.* This method described

by Hamilton and Rowe (85,175) has the advantage of simplicity. A dog is deeply anesthetized with Chlorethane, the carotid artery is cannulated and connected with a mercury manometer, and injections of the extract are made into the femoral or jugular vein. Comparison of the rise in pressure after the injection of definite doses of known and unknown extracts permits estimation of the potency of the latter. Consistent discrimination between doses differing by 10% has been claimed. The published tracings do not appear to support this, though 10% accuracy would be attainable by a sufficient number of test doses.

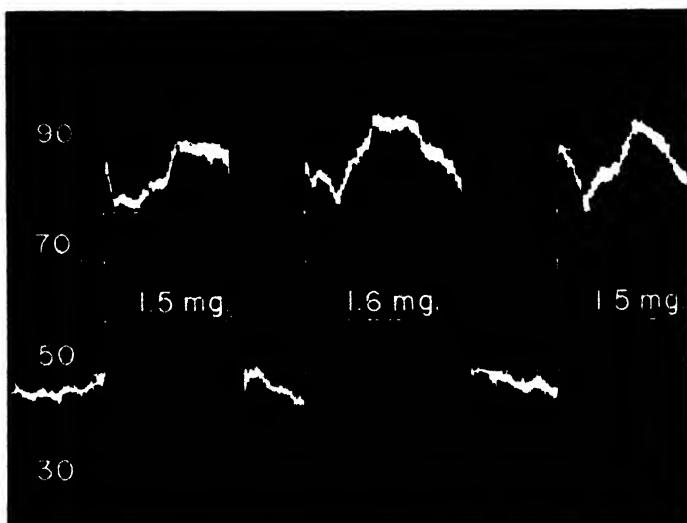


FIG. 2.—Seven per cent discrimination after thirteen previous injections at hourly intervals. Sixteen hours after operation (106).

*b. Injection of Extracts into Spinal Cats.* This method, originally proposed by Laidlaw and Dale, and subsequently improved by Hogben *et al.* (106) in 1924, is more accurate, as judged by published tracings, but suffers from the disadvantage of requiring large uncastrated male cats. It is also time-consuming. The cat is anesthetized with ether, its brain is destroyed, and respiration is given artificially. The procedure is otherwise the same as with the dog. A good preparation discriminates between doses differing by 10%; and 7% discrimination has been obtained (Fig. 2). 25% discrimination can be expected in normal routine work but some preparations are very insensitive and/or discriminate only to about 50%.

*c. Injection of Extracts into Anesthetized Rats.* This method, recently described (121), has several advantages over the others. It is quicker, cheaper, more sensitive, more reliable, and not interfered with by the

presence of histamine (Fig. 3), which is a common contaminant of posterior-lobe extracts. The rat is anesthetized with a barbiturate and the posterior cord is pithed; otherwise the procedure is similar to that described above. This preparation readily discriminates between doses differing by 20% and with care 10% discrimination or less can be achieved (Fig. 3). One of the greatest advantages of this method for routine work is that almost all preparations discriminate between doses differing by 20%.

Anesthetized or spinal cats, dogs, rats, and guinea pigs show this response to whole extract and to pressor fraction. They do not react to the oxytocic

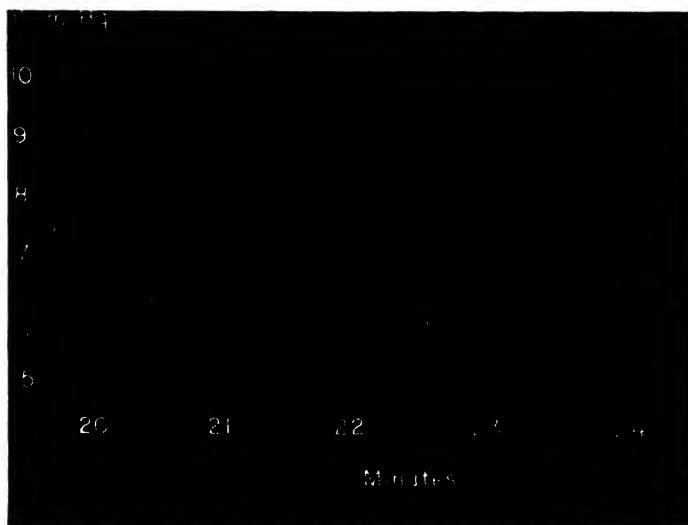


FIG. 3.—Anesthetized rat. Twelve-minute interval (121). Nos. 20, 21 received 0.0096 I.U. posterior-lobe extract; 22, 0.0077; 24, 0.0089. Number 23 received 0.0077 I.U. plus 0.0002 mg. histamine acid phosphate.

fraction (page 461) in this way. It must be emphasized that the ability to bring about the two responses under the conditions prescribed is the *real definition* of pressor and oxytocic activity, respectively, and extracts have been fractionated on this basis.

## 2. Assay of Oxytocic Activity

Two methods are in use for the assay of this activity. The first, initially described in 1912, and subsequently improved by Burn, is sensitive and accurate, but suffers from the serious drawback that only experienced technicians can be relied upon to use it. The uterus is dissected from a virgin guinea pig, immersed in isotonic saline, and connected to a lever writing on a smoked drum. Definite measured quantities of known and

unknown extract are added to the saline, and the degree of contraction of the uterus evoked by each addition is recorded. In this way various doses of known and unknown can be compared (Fig. 4). With the saline normally employed (15) the guinea pig uterus reacts to both whole extract and the oxytocic fraction, but not to the pressor fraction. A saline containing less calcium is sometimes recommended for uteri which will not remain quiescent. We do not recommend this since it greatly reduces sensitivity, though it does not alter the differential response to pressor and oxytocic fractions. If, however, magnesium is added to the saline the uterus responds to the pressor fraction as well as to the oxytocic (49). The literature contains numerous references to the difficulties of obtaining

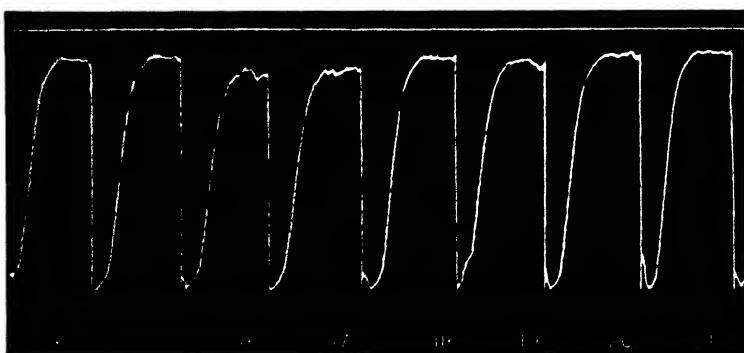


FIG. 4.—Part of an assay for oxytocic activity (225). Doses given at 15-minute intervals. Nos. 14, 15, 18, 19 with 0.035 ml. International-Standard extract; 16, 17 with 0.01 ml. unknown; 20, 21 with 0.015 ml. unknown. Thus 0.01 ml. unknown < 0.035 ml. International-Standard extract, *i.e.*, < 0.07 I.U. and 0.015 ml. unknown > 0.07 I.U. Therefore 0.0125 ml. unknown approx. = 0.07 I.U., *i.e.*, unknown solution contains approx. 5.6 I.U./ml.

consistent results. We have tried two methods of improving the method, *viz.*, substitution of uterine strips from other species and genetic standardization of the guinea pig stock. The former yielded no results of value for the present purpose but the latter has been very fruitful. Taking all the precautions recommended by Burn we frequently had runs of unsuccessful preparations, so we picked litter mates from guinea pigs that yielded good preparations and bred a stock from them. Uteri from this stock have given good results now for several years. An accuracy of 20% can be expected on one preparation by this method and 10% is sometimes obtainable.

The other method depends on the fact that oxytocic activity causes a purely depressor response in the blood pressure of an anesthetized bird. The bird is anesthetized (121), connected to a manometer as in experiments

previously mentioned, and depressor action of known extracts are recorded on a kymograph. The two methods show good agreement provided the pressor-oxytocic ratio does not exceed 2.5. When it does the fowl method yields the higher assays. Smith (22) has suggested that this is due to the high magnesium content of chicken blood and has shown in fact that, if magnesium is added to the guinea pig uterus bath up to 2.5%, the difference between the two methods disappears.

### 3. Assay of Antidiuretic Activity

The method usually adopted for the assay of antidiuretic activity is that of Burn (15). Unanesthetized rats are hydrated by means of a stomach tube, and doses of extract are injected subcutaneously. The delay in diuresis caused by various doses of known and unknown permits the assessment of the strength in the unknown extract. Discrepancy between doses differing by 20% can be obtained with difficulty. Other methods in use are based on intravenous injection into hydrated rabbits (92) or dogs (50). Methods based on intravenous injection are essential when the injected extract contains considerable inert material because the latter slows up the absorption of the activity after subcutaneous injection and so prolongs its effect. A method (83) based on estimation of chloruresis has been described which is claimed to give more consistent results.

## B. PHARMACOLOGY

Administration by mouth of the dried gland or extract produces no obvious effects, while intravenous, subcutaneous, or intramuscular injection of aqueous extracts causes pronounced effects in a number of different organs. The effect of extracts on the isolated organ, however, may be very different from that observed in the intact body. The effect on the intact animal differs to some extent in different species and is greatly modified by anesthesia and mode of administration. The antidiuretic effect, for example, after subcutaneous or intramuscular injection, may be greatly enhanced by introducing substances into the extract or modifying the extract so as to delay absorption of the active principle from the site of injection (22,30,71,176,196). Picrates, tannates suspended in oil, zinc salts, pectin, etc. have been used to delay absorption and increase the duration of the antidiuretic action. This is possible because the kidney notably responds to extremely low blood concentrations of the activity. Such extracts would, however, produce no effects that are obtained only with relatively high blood concentrations.

Most of the effects that have been attributed to posterior-lobe extracts are summarized in Table I. The most clearly established pharmacological properties may be conveniently considered under four heads: cardiovascular

TAB LE I  
Pharmacology of New  
RAW Lobe Extracts

Response	Effects exhibited after injection of ox extract				Excitant in	Phyletic distribution		
	After		After					
	Into un-anesthetized animals	Intra-venous routes	Anesthetized animals	Intra-venous routes				
<b>Mammals</b>								
1. Contraction of uterus smooth muscle	+ 88,108,146	+ 58	+ 24,33,172 168	+ 172,24, 33	Isolated or- gans <i>in vitro</i>	Activity in pituitaries of teleost, amphibian, reptile, bird		
2. Inhibition of water diuresis	+ 153,206 216	+ 153, 206	+ 153, 215	- 153	Other inject. routes	Present in small amount 108, Absent 96		
3. Inhibition of tubular reabsorp-tion	+ 204	+ 204			Spinal animals	Present in small amount 94,92,96 Absent 96		
4. Potentiation of kidney diure-sis	+ 48 + If urine flow low, 58 - 153	+ 48	+ 206 58	+ 206	Intra- venous	Present 103 Absent 97,96 Doubtful 103		
5. Elevation of blood pressure			+ 66,75,165, 105	+ 75,165 + 225	+ 56,24, 105,106 106	- 206		
6. Depression of blood pressure	+ 75,206	+ 75,206	+ 105,126	+ 126	- 105	+ 75,206 - 66 + 56		
7. Coronary constriction	+ 139,206	+ 206, 139	+ 165,1086, 139	+ 165, 1086,139		+ 24,174,1086 + 1086,139 - 108a		
8. Dilation of hepatic veins					+ 108a	- 108a + 108a		
9. Inhibition of coronary con-triction						- 206 + 206		
10. Dilation of coronary vessels						+ 206,77 - 206		
11. Contraction of capillaries	+ 119,120,226	+ 119,	+ 119, 120	+ 119	+ 119	+ 24,120 + 226		
12. Inhibition of secretion of gas-tric juice particularly HCl by stomach wall	+ 29	+ 29						

With well known responses reference is given to easily available works, not necessarily the original.

TABLE I

—Continued

Response	Effects exhibited after injection of				Excitant in		Phyletic distribution	
	ox extract		After		Isolated or glands <i>in vitro</i>	Pressor fraction	Oxytocic fraction	Activity in pituitaries of teleost, amphibian, reptilian, bird
	After	After	Anaesthetized animals	Intra- venous				
Into un- anaesthetized animals	Other inject. routes	Other inject. routes						
13. Increase of blood sugar (hy- perglycemia)	+ 63	+ 63	+ 206	+ 17 Of short duration only	+ 17			
14. Stimulation of intestinal musculature	+ 141,78,142	+ 141, 78 142	+ 78	+ 55,24,141 142	+ 55,24, 141 142	+ 7 (Just Pituitary extract.)	+ 55,24,141 142	+ 55,103, 141 in anesth. dogs only + 142 on rabbits
15. Increase rate of flow of milk from lactating mammary gland	+ 177,56,156, 202, 41	+ 178, 202	+ 177, 86	+ 96,178	+ 96			
16. Facilitation of sleep	+ 181		+ 181?					
17. Effect on fat concentration	+ 206		+ 206				+ 206	- 206
18. Reduction in lymph and edema fluid production	+ 206	+ 206	+ 206					
Birds								
1. Inhibition of water excretion through kidneys	+ 94		+ 94?					
2. Depression of blood pressure				+ 55,105, 101,160	+ 55,105, 101,160	+ 105	+ 105	- 108,21,187
3. Stimulating effect on auricles and ventricles of heart, i.e., heart musculature				+ 160	+ 160			+ 21,55,108, 187
Ropilia				+ 9				Present in small amount 108
1. Inhibition of water excretion through kidneys (not neces- sarily same mechanism as in mammal)	+ 94,93,9							

TABLE I  
—Continued

Response	Effects exhibited after injection of				Excitant in ox extract	Phylogenetic distribution		
	After		After					
	Into un-anesthetized animals	Intra-venous routes	Anesthetized animals	Intra-venous routes				
2. Extra renal change in body water concentration	+ 94, - 93, doubt- ful							
3. Slight depression of blood pressure					+ 105 + 105			
Amphibia					+ 10	+ 10		
1. Inhibition of water excretion through kidney	+ 10 Doubt- ful if any. Evidence only sug- gestive 94, 93	+ 10, 93						
2. Facilitation of water intake (through skin)	+ 94, 189, 93	+ 189, 93		+ 206	+ 189 When muscle sub- merged in bath of ex- tract + 189 When tadpoles injected be- fore muscle removed	+ 94 + 189 + 94 Present 94 amount 94		
3. Elevation of blood pressure				+ 105	+ 105			
4. Contraction of capillaries	+ 120	+ 120		+ 120	Present in amphibia 120			

and respiratory, smooth muscle, water and salt metabolism, and miscellaneous.

### 1. *Cardiovascular and Respiratory Effects*

*a. In anesthetized or spinal Mammals.* Extracts injected *intravenously* into anesthetized or spinal mammals cause a rise of blood pressure which is initially slower but more prolonged than that caused by adrenaline (Fig. 5). This pressor effect is not antagonized by adrenolytic agents nor prevented by vascular denervation, so it appears that the pressor action is direct on muscle and is not mediated by adrenergic vasmotor nerves (70). The peripheral vasoconstriction is not simple. Dermal vessels all constrict, but at least in anesthetized and decerebrate cats and rabbits the capillaries

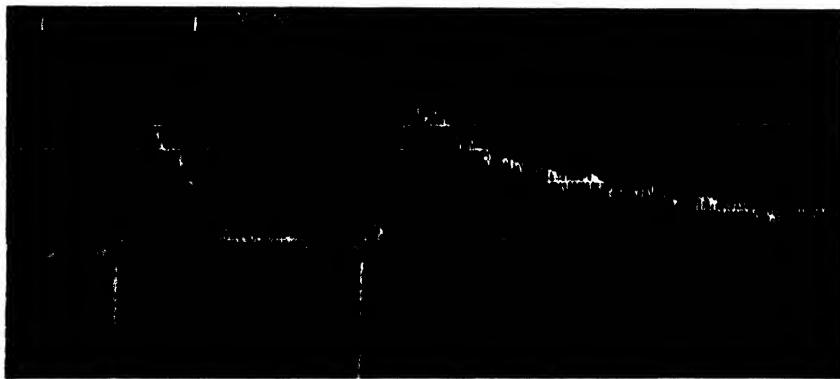


FIG. 5.—Anesthetized rat, continuous trace comparison of the effects evoked by adrenaline and posterior-lobe extract in approx. equipressor doses (225).

in the skeletal muscles may dilate or constrict, so that a redistribution of blood ensues (*e.g.*, Clark, (18), found that the skin, connective tissues, and intestines usually have less, and the muscles more blood after injection). Nevertheless the pressor effect is exhibited by anesthetized rats after ligation of the liver, kidneys, and intestines (225). The coronary artery is constricted and the heart is depressed as a result of diminished blood supply with attendant slowing of its rate, characteristic changes in the T-wave of the electrocardiogram, and sometimes a momentary fall of blood pressure (65). Peripheral vasoconstriction, *per se*, will not increase the blood pressure, so the posterior pituitary must indirectly or directly stimulate heart muscle. Coronary constriction with its attendant effects is inhibited by simultaneous administration of ephedrine and adrenaline. It is also more pronounced after injection of separated pressor fraction

(65) and it seems reasonable to suppose that the oxytocic component inhibits it. The observed coronary dilation after treatment of isolated hearts with oxytocic fraction alone lends some support to this supposition (76). Whether the initial fall of pressure which precedes the rise is *always* directly attributable to coronary constriction is uncertain, but judgment in any case is complicated by two considerations in the *anesthetized* animal. Histamine, a common contaminant of extracts, will evoke a fall. Also, since injections free from histamine cause a fall in unanesthetized animals (below) it is not surprising that they do so if sufficient anesthetic has not been administered. We have frequently observed the depression which Geiling and Campbell (60) have investigated as a property of posterior-lobe extracts in anesthetized animals, but we have always found that deeper anesthesia obliterates it.

With cats, and to a lesser extent with rabbits, tolerance is developed to successive injections, so that diminished pressor responses to equal doses are elicited until a point is reached at which the animal fails to respond. Hogben *et al.* (106) reckoned a 1-hour interval between doses was necessary for complete recovery of sensitivity. Little has been published on the analysis of tolerance and the only information we have about it is that (1) it is a function of dose (not of increment of blood pressure), (2) it is not usually exhibited in the cat when a succeeding dose is injected *immediately* after the blood pressure returns to base (225), (3) experiments involving the Gibb heart show that tolerance is vested in the peripheral tissues (65), and (4) it is not exhibited to any appreciable extent in the anesthetized rat (121) (Fig. 6).

After *intramuscular* or *subcutaneous* injection into anesthetized rats a pressor response is obtained, but not after similar injection into cats and rabbits (225) (Figs. 7 and 8).

b. *In Unanesthetized Mammals.* We know of no records of blood pressure changes after *intravenous* injections of whole extract into unanesthetized mammals, but similar injections of the separated pressor fraction cause a small rise followed by a prolonged depression, which in turn is followed by a maintained rise (76) (Fig. 9). According to Geiling and de Lawder (61): "after intravenous injection of whole extract or pressor fraction into unanesthetized dogs there follows a state resembling anaerobic metabolism in the tissues. During this time the venous blood is characterized by an arterial-like color and oxygen content, a lowered carbon dioxide tension, a rapidly rising lactic acid concentration, increased glucose and inorganic phosphate. This is the period of decreased cardiac output and lowered basal metabolism. Following this stage of tissue asphyxia is the recovery period when conditions are reversed: an abnormally dark venous blood, increased utilization of oxygen and production of carbon dioxide by the

tissues, and a continued rise and gradual return of blood lactic acid to the pre-injection level." These effects are not evoked by Parke Davis' oxyto-

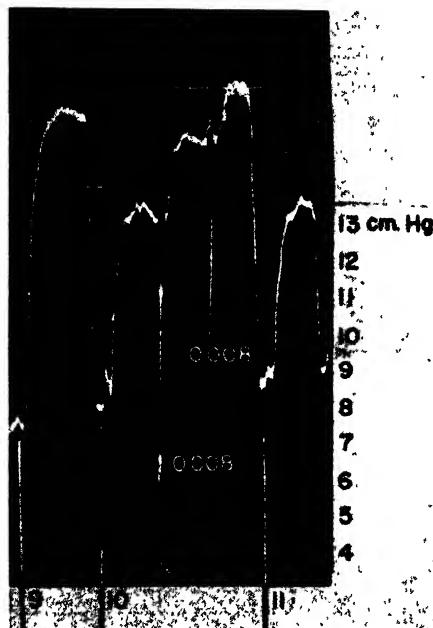


FIG. 6.—Anesthetized rat, showing the absence of tolerance (225). No. 9, 0.024 I.U. posterior-lobe extract at single injection; No. 10, 0.024 I.U. given serially in three doses of 0.008 I.U.; the total increment is the same in both cases. No. 11, 0.008 I.U.



FIG. 7.—Anesthetized rat, continuous trace (225). No. 4, 0.008 I.U. posterior-lobe extract intravenously; No. 5, 1.0 I.U. intramuscularly 15 minutes later; No. 6, 0.008 I.U. intravenously 35 minutes after 5.

cic fraction (pitocin). As with the spinal or anesthetized animal the decreased cardiac output can be attributed to coronary constriction. Parallel observations on anesthetized animals have not been made.

*Intramuscular or subcutaneous* injection of either whole extract or Parke Davis' pressor fraction-Pitressin (man) without anesthesia cause peripheral vasoconstriction without significant increase of blood pressure. There is a brief fall in pulse rate, oxygen consumption, and cardiac output, which is followed by elevation of all three, both after intramuscular (man) or intravenous (dog) injections into unanesthetized subjects.

The action on the heart of unanesthetized animals is variable. Essex (38) *et al.* found that the heart is depressed after injection of extracts in the intact animal, as a result of coronary constriction. Resnik and Geiling (167) studied the cardiac effects of extracts in unanesthetized dogs and observed a short period of acceleration followed by profound slowing of the heart rate, after intravenous injection.

Reflex slowing of the heart rate also occurs after posterior-lobe extract is injected into unanesthetized animals or man. This compensatory mech-



FIG. 8.—Effect of 100 I.U. posterior-lobe extract intramuscularly and 0.05 I.U. intravenously on a decerebrate rabbit, given artificial respiration (225).

anism offsets the effect on blood pressure of peripheral vasoconstriction. These reflexes are apparently sufficiently depressed by anesthesia to allow a rise in blood pressure to occur, but when the extract is administered to normal subjects no rise in systemic blood pressure is produced (153).

In fact, Kolls and Geiling (119) found that a fall in systolic blood pressure occurred in unanesthetized dogs despite capillary constriction. The effect of intramuscular injection of posterior-pituitary extract in man has been studied by Grollman and Geiling (74), who found that no rise in blood pressure occurred. They found that, immediately after injection, the heart rate, oxygen consumption, and cardiac output were temporarily diminished. The venous blood became arterial in character, and they concluded that both cardiac and peripheral effects combined to interfere with the oxygen supply to tissue; oxygen debt followed and a more lasting increase in cardiac output and oxygen consumption subsequently occurred to compensate for this debt.

The effect on the heart of posterior-lobe extracts injected intravenously

into *anesthetized* mammals is complicated by the direct action of the extract on coronary constriction. This is conditioned by the species, the degree and kind of anesthesia, and the quantity and type of extract. Some measure of coronary constriction probably always occurs and sometimes the effect is sufficiently pronounced to cause either a pure fall in pressure (due to heart failure) or a secondary fall after the initial rise. Although much work has been done (137,140,165,175) insufficient is known for a complete analysis of the situation. A significant observation (117) is that in *isolated* hearts oxytocic fraction causes vasodilation and that whole extract does not evoke the degree of coronary constriction evoked by the pressor fraction alone. We are unable, however, to find a clear statement that this state of affairs holds after injection into anesthetized animals.

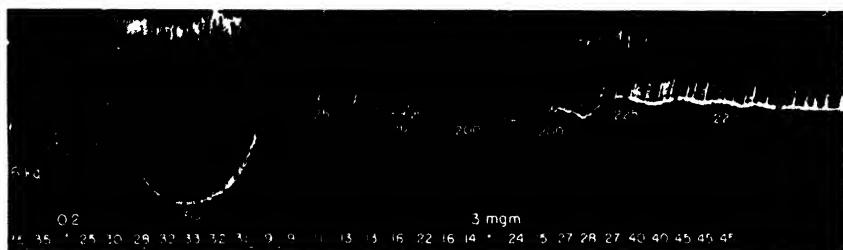


FIG. 9.—Effect of vasopressor principle (Pitressin) on circulatory system and respiratory movements of the dog (local anesthesia only) (76). Top record: respiratory movements (pneumograph and tambour); middle record, blood pressure (membrane manometer; figures indicate pressure in mm. Hg); bottom record, time in 15-second intervals (figures indicate number of heartbeats per 15-second period). At ↑ 0.2, 0.2 ml. Pitressin given intravenously (2/3 unit/kg. body weight). At ↑ , 3 mg atropine sulfate given intravenously.

*Respiratory movements* are secondary to circulatory effects. In unanesthetized animals there is a quickening of the respiratory rate, interspersed with periods of cessation of breathing (65). A tracing by Gruber and Kountz (76) (Fig. 9) shows the time relations between these respiratory effects and the vascular changes.

Information about the effects of posterior-lobe extract on the vascular system of lower vertebrates, with the exception of birds, is meager. Pitressin has little effect on the branchial vessels of the teleost *Anguilla* (117) and, whereas we found a marked effect on the amplitude and rate of both the heart and breathing movements of elasmobranchs (225), there was no great increase of blood pressure. Frog capillaries are constricted by pituitary extract but enormous doses are required to elevate the blood pressure of a pithed animal (21,105,160). The prominent effect on the blood pressure of the tortoise is depressant and intravenous injection into

anesthetized or spinal birds produces a fall in blood pressure (page 436) due to dilation of the systemic arterioles (21,105,160).

## 2. Smooth-Muscle Effects

Whole pituitary-lobe extracts cause contraction of smooth muscles. Most work has been done on the uterus and alimentary canal. Observations on the uterus of different species *in vivo* and *in vitro* have not been sufficiently correlated to permit a general synthesis. *In vitro* studies with guinea pigs show that a uterus removed at diestrus (or castrate) has little spontaneous activity in the saline specified for assay purposes (15), and reacts both to whole extracts and the oxytocic fraction but not to pressor fraction. Both its spontaneous action and response to pituitary are reduced by decrease in calcium ions in the saline (169). With the addition of magnesium to the bath the uterus becomes sensitive to the pressor fraction (49). Contrary to early reports, addition of estrogen to the bath will not potentiate the effect of pituitary extracts. The rat uterus *in vitro* shows spontaneous contractions irrespective of the phase of the donor. Its sensitivity to extracts is not conditioned by the sex phase. Its response to calcium and magnesium in relation to pituitary extracts and separated pituitary fractions is similar to that of the guinea pig (225). The rabbit uterus *in vitro* is responsive to both pressor and oxytocic fractions, but we are unaware of any work on the ionic concentration of the bath suggested by this. Uterine strips taken during pregnancy or pseudo-pregnancy (*i.e.*, luteal phase) show decreased sensitivity to pituitary extracts, but this sensitivity is regained toward the end of pregnancy (169). Contradictory reports have appeared about the behavior of the human uterus *in vitro* (144) and our experience with it indicates that it is affected by both fractions at all phases of the sex cycle.

*In vivo* studies on the mouse and rabbit show that in the estrus phase and toward the end of pregnancy the uterus is very sensitive to posterior-lobe extracts and is insensitive during the luteal phase. Increased sensitivity of the pregnant mouse uterus can be induced by previous estrogen injections (169). Morgan (146) has analyzed the effect of extracts on the uteri of unanesthetized rabbits. The oxytocic fraction caused contraction followed by augmented activity, and the contraction due to the pressor fraction was followed by a period of lessened activity accompanied by a fall in volume of the uterus due to a spasm of its vessels. The effect of whole extract was equivalent to the sum of its two components. The consensus with regard to the human uterus *in vivo* is that during early stages of pregnancy the uterus is responsive to the pressor (vasomotor effect?) but not to the oxytocic fraction. During parturition the uterus is very reactive to the oxytocic fraction and to whole-pituitary extracts,

though the effects appear to be qualitatively different. In the nongravid uterus the pressor is more effective than the oxytocic fraction in eliciting contractions (65).

The action of posterior-lobe extracts on the intestinal tract has been studied both on isolated strips and *in vivo*. Gruber and Robinson (78) observed inhibition of the intestine in unanesthetized dogs after administration of whole extracts, and pressor and oxytocic fractions, though the last was not so powerful. Gaddum (56) found that both pressor and oxytocic fractions produce shortening of an isolated loop of rabbit intestine and an increase of peristalsis. He found the colon to be most sensitive, the ileum and the duodenum least; the pressor fraction was more effective than the oxytocic. This was confirmed by Melville and Stehle (141) using isolated loops of rabbit intestine but they obtained different results after intravenous injection into the intact dog. Both fractions cause a defecation in the unanesthetized dog but different and characteristic actions are produced on the bowel. The pressor fraction stimulated the small intestine and colon; peristaltic contractions were marked and the small intestine contents were expelled into the large bowel. The oxytocic fraction did not affect the colon, but relaxed the circular muscle and constricted the longitudinal muscle of the small intestine. None of the contents of the small bowel passed the ileocecal valve.

The effects on the human bowel were studied by Guthrie and Bargen (82), who concluded that whole posterior-pituitary extract is a powerful stimulant of intestinal peristalsis, acting on both the colon and ileum. It increases the motility of the intestine without apparently affecting tonus. Broadly speaking, extracts with a high pressor titer cause an increase, and extracts with high oxytocic content cause a decrease in intestinal tone.

The other smooth-muscle effect studied is the galactogenic action (40). It increases temporarily the amount of milk obtainable from lactating animals by stimulating the smooth muscle of the ducts of the mammary glands, resulting in a more complete expression of their contents. It has no effect on total milk production over extended periods of time.

### 3. Water and Salt Metabolism

The effect of posterior-lobe extracts on water and salt metabolism varies according to the test animal and the experimental conditions. The first effect discovered was a diuretic action, now considered a secondary effect, and one which can only be observed under special conditions.

Intravenous injection of large doses of whole extracts or of pressor fraction into anesthetized animals produces an initial anuria followed by a definite diuresis of hydrated animals (130). The urine from the diuretic period contains increased amounts of chloride both relatively and abso-

lutely. The diuretic action is believed to be a secondary effect due to increased filtration pressure at the renal capillaries as a result of the pressor action of the extract (70).

Intravenous or subcutaneous injection of small doses of whole extracts or of pressor fraction into unanesthetized hydrated mammals has an antidiuretic effect. The urine collected during the antidiuretic period contains an increased amount of sodium, chloride, phosphate, and total nitrogen, so that, despite the decrease in volume, more chloride is excreted in unit time. The effect is exhibited by both denervated and isolated kidneys, hence its seat of action is the kidney itself (70). Phyletic evidence has localized the seat of reabsorption in the loop of Henle. The antidiuretic effect is attributed to increased reabsorption of water by the tubular epithelium; the glomerular filtration rate appears to be unaffected (91). Whole extract inhibits a water diuresis but has no effect on a salt-induced diuresis, and additional extract has no effect on a normal kidney already excreting a maximally concentrated urine (12). The extract also decreases the resorption of salt by the tubular epithelium. Subcutaneous administration of the oxytocic fraction, however, in small doses has been found to produce water and salt diuresis in unanesthetized rats (but not dogs), and the pressor fraction antagonizes this (50) (Table 1). Schutz (181) has recently shown that humans subjected to increased water load after lunch (*i.e.*, at a time when they were drowsy) were more liable to sleep, and this was greatly facilitated by simultaneous injection of posterior-lobe extracts.

It is of interest to note that adrenalectomy is followed by increased urinary sodium so that in this respect it resembles the condition after injection of posterior-lobe extract. Injection of desoxycorticosterone acetate produces effects of water and salt metabolism which resemble *diabetes insipidus*. On the basis of these findings a balance between the activity of the adrenal cortex and posterior lobe in the intact animal is suspected (110).

Amphibia are the only other group of animals extensively investigated in this context (94). No experiments have been reported on the effect of intravenous injection into anesthetized amphibia and an antidiuretic effect in unanesthetized animals is doubtful. However, injection of whole extract into frogs in water leads to increased body water due to intake through the skin. Heller's studies have shown: (a) this effect is obtained with extracts of glands from all classes of vertebrates that have a *pars nervosa*; (b) it does not parallel the oxytocic and pressor potency of extracts but is found in greater concentration in the oxytocic than in the pressor fraction of ox pituitary extracts, and (c) the effect is exhibited more strongly by amphibia inhabiting drier habitats than by more nearly aquatic forms.

Information about other vertebrates is meager. In unanesthetized birds there is increased reabsorption of water and diminished glomerular filtration. In alligators, the only effect is decreased glomerular filtration. No antidiuresis was observed in the fish *Ameiurus*. These facts led Burgess *et al.* (12), Heller (91), and Marshall (131) to conclude that posterior-lobe extract caused reabsorption of water via Henle's loop, which is present in birds and mammals but not elsewhere.

#### 4. *Miscellaneous Effects*

Many other effects of administration of posterior-lobe extract have been reported. The extract may affect carbohydrate metabolism by (a) producing hyperglycemia, (b) preventing the hypoglycemic action of insulin, and (c) antagonizing the hyperglycemic action of adrenaline. Stehle's pressor fraction elevates the blood sugar of rabbits more than his oxytocic fraction, while the latter has a greater effect in the dog than has the former (36,108). Kamm's pressor fraction actually reduces the blood sugar of the rabbit (81). Thus the hyperglycemic effect after intravenous injection of posterior-lobe extracts may be associated with either the pressor or oxytocic fraction according to the method of preparation and according to the species used as test animals. This, together with the fact that large doses are required, indicates that the effect is due to some nonspecific component of the extracts. This view is reinforced by the fact that van Dyke's purified protein has no effect on the blood sugar of rabbits, and it is improbable that the neural lobe is significant in carbohydrate metabolism.

The anti-insulin effect reported by Burn (13) was investigated by Griffith (73) who concluded that it was due to peripheral vasoconstriction delaying absorption of subcutaneously injected insulin. Ellsworth (36,108), on the other hand, found that the oxytocic fraction antagonized insulin hypoglycemia in the dog when both were injected intravenously. Young (227), however, was able to prevent the action of insulin by intravenous injection of anterior-lobe extracts into rabbits and it would appear that this effect is not a specific property of neural-lobe extracts.

The antiadrenaline effect of whole extracts is associated with the melanophore-dispersing fraction and is probably not associated at all with the neural lobe (page 501).

Inhibition of gastric secretion after treatment with histamine and other stimuli is probably due to the vasoconstrictor action of the pressor fraction on the blood vessels supplying the glandular secreting cells. Other effects, such as removal of edema fluid, production of anemia, and production of fatty changes in the liver are also probably effects due to the pressor fraction (206).

### C. PURIFICATION AND CHEMISTRY

As each of the various effects of crude neural-lobe extracts was discovered, there was a tendency to assume that each effect was due to a different substance in the extract. As a result, most of our information regarding the purification and chemistry of the activities comes from efforts to decide how many substances must be present in the crude extract to account for its various effects. This problem is still unsolved and the evidence is analyzed in Section III, D. Most of the literature is concerned with the purification and fractionation of the pressor and oxytocic activities as defined by the methods of assay previously outlined. It is appropriate that we here group our information according to whether the end product contains pressor and oxytocic properties in equal or different proportions.

#### 1. *Unfractionated End Products*

The procedure of dehydrating and defatting fresh glands by treatment with acetone, first used by Smith and McClosky (184,185) and now a standard procedure, yields, if carefully done, a product containing properties approximating to the International-Standard Powder (I.S.P.). Over the last few years we have made five substandard powders by this method for our own use and have produced powders always between 80 and 90% as potent as I.S.P. when measured for oxytocic and pressor activities by the standard methods. Considerable inert protein can be removed from this powder by simple procedures such as acetone precipitation from acidified extracts. We have, however, found it difficult to obtain a final product more than seven times as potent as I.S.P. without some fractionation. Most workers interested in further purification have fractionated their extracts and have not given figures for the purity of their intermediate products. It is true that Abel (1) claimed to have achieved a high degree of purification without fractionation but reasons are given elsewhere for doubting the validity of his conclusions. MacArthur (129), by precipitation of the material from acidified methanol extracts, obtained a purified product with both pressor and oxytocic properties, but no figures for purity were given and his work was never followed up. His product was sparingly soluble in water and had an isoelectric point at about pH 5. Its potency was destroyed by acid hydrolysis, weak alkali, and trypsin, and it contained labile sulfur.

Chief interest now centers on recent well documented information given by van Dyke *et al.* (207). These workers isolated from the posterior lobe a noncrystalline protein which appears to be homogeneous and which contains pressor and oxytocic properties in the same ratio as in I.S.P. (see page 432). It is almost devoid of melanophore-expanding activity.

Their initial extract was made from fresh glands with dilute sulfuric acid and purification of this by precipitation with sodium chloride in acetate buffer was carried out until constant solubility of dissolved nitrogen

TABLE II (113)  
PURIFICATION PROCEDURE OF VAN DYKE AND COLLABORATORS

Fraction	Procedure	Potency, units/mg.		Total ac- tivity, %	
		Pres- sor	Oxy- toxic	Pres- sor	Oxy- toxic
A	Fresh posterior lobes (1 kg.) Ground and suspended in 0.01 N H <sub>2</sub> SO <sub>4</sub> , pH 4.25 ↓	0.2	0.2	100	100
B	Supernatant from centrifuging pH adjusted to 3.9; 80 g. NaCl/l. added ↓	—	—	75-80	75-80
C	Precipitate Dialyzed Cl <sup>-</sup> free; 10 g. NaCl/l. added pH 3.5 ↓	—	—	50	50
D	Supernatant from centrifuging Equal vol. 1 M acetate buffer, pH 3.9 added; 20 g. NaCl/l. added ↓	—	—	—	—
E	Supernatant from centrifuging and wash- ing. 40 g. NaCl/l. added ↓	—	—	—	—
F	Precipitate Dissolved in minimum vol. water; equal vol. 1 M acetate buffer added; 6.5 g. NaCl/100 ml. added ↓	—	—	—	—
G	Precipitate Previous step repeated until dissolved N is constant (0.100 mg./ml. at 25°C). ↓	—	—	—	—
H (Active protein)	Precipitate (700 mg.)	17	17	6	6

resulted (see Table II). 1 kg. dried posterior lobes yielded 700 mg. amorphous protein containing 17 units/mg. (*i.e.*, 8.5 × I.S.P.). Its molecular weight judged by ultracentrifugation is 30,000 and calculated

from the amino nitrogen value (assuming 1 amino group/molecule) is 26,000. Table III shows the chief properties of the protein. It exhibits a solubility curve typical of a pure substance, sediments in the ultracentrifuge as a pure protein, and assays of various fractions from solubility, electrophoresis, and ultracentrifuge tests give no indication of the presence of potent contaminants of different characteristics.

Constant solubility was obtained with the protein in 0.5 M acetate

TABLE III (113)  
PROPERTIES OF THE POSTERIOR LOBE PROTEIN

Activity, units/mg.	
Pressor . . . . .	16.6
Oxytocic . . . . .	16.6
Antidiuretic . . . . .	16.4
Elementary analysis, %	
Carbon . . . . .	48.64
Hydrogen . . . . .	6.63
Nitrogen . . . . .	16.32
Amino nitrogen . . . . .	0.054
Phosphorus . . . . .	0.027
Sulfur . . . . .	4.89
Chlorine . . . . .	0.02
Ash . . . . .	0.58
Oxygen (by difference) . . . . .	22.89
Sulfur distribution	
Cysteine . . . . .	0
Cystine . . . . .	4.3
Methionine . . . . .	?
Sulfate . . . . .	0.1-0.4
Molecular weight (ultracentrifuge) . . . . .	30,000
Isoelectric point (electrophoresis) . . . . .	4.8

buffer at pH 3.9 containing 6.5% sodium chloride. Throughout the solubility curve the dissolved pressor and oxytocic activities were found to be equal and constant. The potency of a sample of the pure protein was unchanged after three washings with the above solvent even though 54% of the total activity was removed in the process. The supernatant fluid from a suspension of the protein in this solvent showed the same ratio of pressor and oxytocic potency as the protein itself.

Ultracentrifuge studies in the pH range 3.30 to 3.84 indicated homogeneity of the protein by the symmetry of the sedimentation curves

and by the absence of significant displacement of the base line. After ultracentrifugation, almost 100% of the pressor and oxytocic activity was found to sediment with the protein. The sedimentation constant was found to be 2.61 to 2.8 Svedberg units, and assuming a specific volume of 0.749 a molecular weight of 30,000 was calculated.

The electrophoretic mobility was studied over a pH range 3.4 to 6.2 and the isoelectric point estimated to be pH 4.8. Between pH 3.41 and 3.47 the electrophoretic pattern showed one main component but a second component, present in very small amount, also appeared. The potencies of the different fractions (main component, "protein-free solution," and the minor component) were indistinguishable on the basis of nitrogen. It was suggested that the minor component either derived from or was closely related to the main protein component. At all other pH values in the range studied electrical inhomogeneity was found but no second component could be discerned. In spite of this, assays performed upon various fractions of the electrophoresis cell failed to reveal the presence of substances of greater or less potency than the protein. Van Dyke *et al.* believe that strong evidence has been adduced in favor both of the homogeneous nature of the protein and the presence of its properties as integral parts of the protein, but they point out that their data do not preclude the possibility that the protein itself is pure and is pharmacologically active by reason of small, active, separate substances adsorbed to it.

Irving and du Vigneaud (113) criticized van Dyke's findings and have suggested further tests which would help to discriminate between these possibilities. They point out that solubility tests have been made only in a solvent identical in pH and composition with that used in attaining constant solubility in the final steps of the isolation procedure, and that the subsequent determination of solubility under the same conditions would be expected to furnish no additional information regarding purity. Electrical inhomogeneity was found in electrophoresis experiments in which the pH of the medium was varied between 3.4 and 6.1, whereas no evidence of inhomogeneity was observed in solubility and ultracentrifuge experiments when the pH was allowed to vary only from pH 3.3 to 3.9. Irving and du Vigneaud suggest that when sufficient amounts of the protein are available, solubility curves, together with assays for each of the activities at different points in the solubility curve, should be carried out at several pH values. If the active material can be dissociated from the protein, it should be possible to isolate the inactive protein. Suitable amounts of the separated principles could then be added to it and the product tested for constant solubility and activity under the conditions used in the original isolation procedure. This might indicate whether the protein is a pure, active substance which is fragmented by the treatments employed, or a protein

whose activity is simply adsorbed or eluted depending upon the conditions of the solvent. If dissociation of the protein and activity cannot be accomplished, known amounts of the separated active principles could be added to the protein and the solubility curve and the activity at different points on it could be studied. If activity is actually adsorbed a product might be isolated having a pressor/oxytocic ratio greater than unity by precipitating the protein from a solution to which a purified pressor preparation has been added.

Ultracentrifuge experiments with the active protein should also be tried at widely different pH values. The behavior of the active protein together with known amounts of the separate principles should be investigated in the ultracentrifuge under the conditions reported for the protein alone, to discover whether or not the added, low-molecular principles sediment with the protein.

Irving and du Vigneaud also suggest electrophoretic separation of the components under conditions such that sufficient active material is present in each of the fractions to afford complete and accurate assays for all the activities. They consider that the electrical inhomogeneity observed at certain pH values may be an indication of the separation of protein and adsorbed active principles.

A significant difference between the separated pressor and oxytocic extracts and van Dyke's active protein is found *when the protein is treated with cysteine*. Sealock and du Vigneaud found that cysteine reduction of separated pressor and oxytocic extracts caused no loss of pharmacological activity (182). On the other hand, van Dyke and co-workers find that treatment of their protein with cysteine causes a 44% loss of activity after 2 minutes and 80% loss after 5 minutes. These observations indicate that the active protein is not merely an inactive substance with adsorbed active principles. If these results can be confirmed under strictly controlled conditions the procedure should be repeated on a mixture of the protein and the separated principles to determine whether the presence of protein affects the reduction.

It is obvious, therefore that much work needs to be done before we can accept the protein as a hormone of the neural lobe possessing multiple activities.

## 2. Fractionated End Products

Two methods have been used for fractionating and purifying extracts, (a) differential precipitation from organic solvents, and (b) differential adsorption and elution.

a. *Fractional Precipitation.* This method depends upon the solubility of the active principles in aqueous solution and their differential solubility

in organic solvents and in concentrated salt solution. Dilute acetic acid is usually employed for the initial extraction, since the pH of the solution

TABLE IV (113)  
PURIFICATION PROCEDURE OF KAMM AND CO-WORKERS

Fraction	Weight, g.	Procedure	potency, units/mg.		Total ac- tivity, %	
			Pres- sor	Oxy- toxic	Pres- sor	Oxy- toxic
A	1000	Acetone-desiccated posterior lobes  Extracted with hot 0.25% HAc; concentrated; saturated with $(\text{NH}_4)_2\text{SO}_4$ .  ↓	1	1	100	100
B		Precipitate, dried  Extracted with glacial HAc; ether and petroleum ether added to extract.  ↓				
C	68	Precipitate  Dissolved in 98% HAc; ether added; repeated once.  ↓	11	12	75	82
D	63	Precipitate  Dissolved in 98% HAc; fractionally precipitated with acetone and ether to give several precipitates.  ↓	10	7	63	44
E	5.9	Most potent precipitate  Dissolved in 98% HAc; fractionally precipitated with acetone and ether as above.  ↓	62	14	37	8.3
F (Pressor)	2.0	Most potent precipitate  ↓  HAc-ether mother liquor treated with trace of water and excess of petroleum ether.  ↓	80	15	16	3.0
G (Oxytoxic)	2.4	Gummy precipitate	6	160	1.5	38

approximates that of maximum stability of the activities (pH 3.0). Three methods have been extensively employed, and the procedures are sum-

marized in Tables IV-VI, taken from papers of Irving and du Vigneaud. For comparative purposes, the yields have been recalculated to show the amounts of purified pressor and oxytocic fractions which can be obtained from 1 kg. dry posterior lobes or its equivalent in fresh glandular tissue. The figures in Table VII indicate that the most potent pressor preparations

TABLE V (113)  
PURIFICATION PROCEDURE OF STEHLE AND FRASER

Fraction	Weight, g.	Procedure	Potency, units/mg.		Total activity, %	
			Pres- sor	Oxy- toxic	Pres- sor	Oxy- toxic
A	1000	Acetone desiccated posterior lobes Extracted with hot 0.5% HAc; concentrated; treated with absolute ethanol. ↓	1	1	100	100
B	218	Alcohol precipitate Dissolved in 0.5% H <sub>2</sub> SO <sub>4</sub> ; Ba(OH) <sub>2</sub> , Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> and dialyzed iron added; filtrate concentrated to dryness; dissolved in 96% methanol; fractionated with EtAc, repeated twice. ↓	4	4	87	87
C	7.3	Precipitate Fractionated from ethanol or methanol with EtAc. ↓	100	30	73	22
D (Pressor)	2.0	Precipitate ↓ Methanol-EtAc mother liquor fractionated with methanol, ethanol, and EtAc. ↓	200	10	40	2
E (Oxytocic)	0.55	Precipitate ↓	4	250	0.2	14

also contain small amounts of oxytocic activity. Similarly, the oxytocic preparations contain small amounts of pressor activity. Since it is possible to fractionate partially a crude extract in terms of its action on the isolated guinea pig uterus and on the blood pressure of an anesthetized animal, many workers have lapsed into the habit of thinking operationally as

**TABLE VI (113)**  
**PURIFICATION PROCEDURE OF IRVING, DYER, AND DU VIGNEAUD**

Fraction	Weight, g.	Procedure	Potency, units/mg.		Total activity, %	
			Pres- sor	Oxy- toxic	Pres- sor	Oxy- toxic
A	6000	Frozen posterior lobes Ground with sand and water; juice separated under pres- sure; juice acidified, boiled 10 min., centrifuged; super- natant concentrated; satu- rated with NaCl. ↓	0.2	0.2	100	100
B	200	Precipitate, dried Extracted with 98% HAc; ether and petroleum ether added to extract. ↓	4	4	80	80
C	78	Precipitate Dissolved in 98% HAc; ether added; repeated once. ↓	9	9	72	75
D	58	Precipitate Dissolved in 98% HAc; frac- tionally precipitated with acetone and ether to give several precipitates. ↓	9	5	52	27
E	5.2	Most potent precipitate Dissolved in 98% HAc; frac- tionally precipitated with acetone and ether as above. ↓	55	30	29	16
F	1.5	Most potent precipitate Dissolved in glacial HAc; precipitated with ether; re- peated 3 times. ↓	85	30	13	4.5
G	1.1	Precipitate Dissolved in water; extracted with butanol. ↓	110	—	12	—
H	0.6	Aqueous solution Electrophoresis and isolation of cathode material. ↓	125	20	8.1	1.3
I (Pressor)	0.2	Precipitate ↓ HAc-ether mother liquor treated with water and petro- leum ether. ↓	200	26	4.0	0.5
J (Oxytoxic)		Gummy precipitate	22	146	5	34

though there are two activities in the original material, an "oxytocic" substance and a "pressor" substance. These terms are put in inverted commas because they have not the same meaning as the words pressor and oxytocic as ordinarily used, though some workers do not appear to appreciate the difference. They have lost sight of the fact that the terms as ordinarily used are defined by the assay method. If we assume that these are the two substances "pressor" and "oxytocic" we can say that the

TABLE VII (110)  
ANALYSIS OF PRESSOR AND OXYTOCIC PREPARATIONS

Activity, units/mg.		Analysis, %						Ref.	Remarks
Pressor	Oxytocic	N	S	NH <sub>2</sub> -N	Cystine	Tyrosine	Arginine		
200	?	—	3.1	—	—	10.5	—	214	Values corrected for ash and moisture.
?	500	—	3.1	—	9.0	14.3	—		
200	6	15.6	3.4	—	—	11.5	3.8	182	Values corrected for ash and moisture.
2	500	15.2	3.3	—	9.6	15.8	—		
200	8-10	13.9	3.0	2.0	7.7	9.5	8.9	194	Moisture and ash corrections not indicated. Average values.
4	250	13.8	3.6	2.5	8.9	10.7	6.1		
4	4	13.7	1.6	1.2	—	2.6	8.6		
200	25	14.3	—	—	11.2	9.9	—	112	Not corrected for ash or moisture
9	9	14.6	—	—	10.8	2.2	—		
?	240	14.0	3.2	1.4	—	—	—	53	Moisture and ash corrections not indicated.
450	<40	—	—	—	19.0	11.9	12.3		
<20	700	—	5.6	—	18.3	14.2	<0.8	164	Moisture and ash corrections not indicated.

former is predominately pressor and the latter predominately oxytocic when tested by the orthodox assay methods. The fact that the purest preparations contain both activities is not important on this assumption. It *may* be, for example, that the guinea pig uterus is very sensitive to the "oxytocic" substance and less sensitive to the "pressor" substance. It is interesting to speculate on a possible species variation which determines how a crude posterior-lobe extract is most readily split into two parts. Existing purified extracts, made from ox pituitaries, might be completely separated if tested on bovine tissue, and similar manipulation of rabbit pituitary material might give two quite different extracts.

Stehle (191) has reviewed fully early attempts at fractionation of posterior-lobe extracts. The first method to result in end products substantially free from the other activity was that published by Kamm *et al.* in 1928 (115). They prepared a pressor fraction containing 160 units/mg. and an oxytocic fraction containing more than 300 units/mg. Each was contaminated with 2-5% of the other property. Kamm prepared extracts with an oxytocic content of 500 units/mg. The procedures employed by Stehle (194) and Irving (112) yield pressor and oxytocic preparations each containing about 250 units/mg., but the yield is low (about 0.1% of the original). The purified pressor and oxytocic preparations made by differential precipitation are stable, white, amorphous powders.

*b. Fractional Adsorption.* Various attempts have been made to separate pressor and oxytocic properties by adsorption methods. A German patent (109) describes how the former is more readily adsorbed on bentonite and may be eluted with weak alkali. We found that pressor activity was more readily adsorbed on charcoal from a simple crude extract and could be eluted with phenol or glacial acetic acid while the oxytocic fraction could be salted out from the residual mother liquor. A high yield (20-40%) but not a high degree of purity (20-100 units/mg., 10-70% contamination) was obtained and the method was found to be unreliable. Gulland (80) *et al.* used Norit in an attempt to further purify the oxytocic fraction of Kamm *et al.* but were only partially successful.

Potts and Gallagher (163,164) used artificial zeolites for adsorption of the pressor fraction and eluted it with salt solution. In this way they obtained the most potent preparations yet described (see Table VII).

### 3. Physical and Chemical Properties of the Fractions

Schaefer and Vincent (179) first found that pressor activity will pass through a dialyzing membrane and this has since been found to be true also for oxytocic and antidiuretic activities. In this way, the size of the molecules responsible for the activities has been estimated. Smith and McClosky found that all activities pass through a collodion membrane impermeable to trypan blue (mol. wt. 932) and the diffusion rate was similar to that of methylene blue (mol. wt. 374). Kamm *et al.* by similar methods estimated the molecular weights of each fraction to be about 600. Stehle and Fraser (194) found that the simplest molecular weights calculated from the sulfur contents of their fractions, assuming the sulfur is present as cystine sulfur, are 1778 and 2160 for oxytocic and pressor, respectively. Potts and Gallagher find that the cystine and tyrosine values for their 700-unit/mg. oxytocic fraction agree with a minimum molecular weight of 1300 for the oxytocic principle, assuming the presence

of one molecule of each amino acid. On this basis, a similar minimum would apply to their 450-unit/mg. pressor fraction. Thus it would appear that the two active fractions have molecular weights between 500 and 2000.

Kamm *et al.* did not investigate the chemical nature of their fractions in their original paper, but du Vigneaud *et al.* (212) later published the results of efforts to purify further the original preparations. They mention an oxytocic fraction (500 units/mg.) which contained 3.06% sulfur, 8.96% cystine, and 14.3% tyrosine. Their pressor fraction (200 units/mg.) con-

TABLE VIII (195)

## IDENTIFICATION OF AMINO ACIDS IN HYDROLYZATES OF PRESSOR AND OXYTOCIC PREPARATIONS

Amino acid	Characterized by	Pressor preparation	Oxytocic preparation
Tyrosine	Picrolonate	+	+
Cystine	Amino acid, hydantoin	+	+
Arginine	Flavianate	+	+
Proline	Reineckate	+	+
Isoleucine	Hydantoin	+	-
Leucine	Hydantoin	-	+
Histidine	Colorimetric test	-	-*
Glutamic acid	Hydrochloride	-	-
Phenylalanine	Picrolonate	-	-
Hydroxyproline	Reineckate	-	-
Glycine	o-phthalic aldehyde	-	-
Tryptophane	Colorimetric test	trace	trace
Cysteine	Colorimetric test	-	-

\* Freudenberg and co-workers (53) found tyrosine, cystine, and histidine in the hydrolyzate of an oxytocic preparation (240 units/mg.).

tained 3.10% sulfur, 10.5% tyrosine, and only a trace of cystine. These data together with similar data obtained by other workers are given in Table VII taken from Irving's review (110).

It is doubtful whether the substances analyzed were pure enough to predict the composition of the principles themselves but the figures indicate that highly purified pressor preparations contain large amounts of cystine, tyrosine, and arginine, while oxytocic preparations contain similar amounts of cystine and tyrosine but much less arginine.

Stehle and Trister (195) have recently examined purified preparations for a large number of amino acids (see Table VIII). Significant amounts of substances other than amino acids have not been found and the "pressor" and "oxytocic" substances are regarded as closely related polypeptides.

There is much evidence to substantiate this view. Positive biuret and ninhydrin color reactions are obtained with purified preparations. Acid hydrolysis and tryptic digestion rapidly destroy the activities with liberation of amino acids. Hydrolysis results in a four- to fivefold increase in

TABLE IX (222)

REACTIONS TAKING PLACE DURING VARIOUS TIME INTERVALS ACCORDING TO THE ONE-HORMONE AND TWO-HORMONE HYPOTHESES

Time interval	One-hormone hypothesis	Two-hormone hypothesis
(1) $wT_b$	<i>Maximum increase of "B" due to release from reflex inhibition by peripheral retinal elements</i>	<i>Simple decrease of "W" while "B" remains constant. "B":"W" ratio increasing</i>
(2) $sT_w$	<i>Maximum decrease of "B" due to reflex inhibition by peripheral retinal elements</i>	<i>Simple increase of "W" while "B" remains constant. "B":"W" ratio diminishing</i>
(3) $sT_d$	<i>Decrease of "B" due to nonstimulation of basal retinal elements</i>	<i>Decrease of "B" due to nonstimulation of basal retinal elements</i>
(4) $sT_b$	<i>Increase of "B" due to stimulation of basal retinal elements alone</i>	<i>Increase of "B" due to stimulation of basal retinal elements alone</i>
(5) $sT_w$	<i>Submaximum decrease of "B" due to stimulation by basal retinal elements and prepotent reflex inhibition of "B" secretion through stimulation by peripheral retinal elements</i>	<i>Concomitant increase of "B", and of "W"</i>
(6) $wT_d$	<i>Submaximum increase of "B" due to simultaneous release from both (a) stimulation of "B" secretion by basal retinal elements and (b) prepotent reflex inhibition by peripheral retinal elements</i>	<i>Concomitant decrease of "B," and of "W"</i>

amino nitrogen. It may be concluded that the preparations contain amino acids joined in peptide linkage and, from the size of the molecule, that they are polypeptides.

Apart from the fact that Potts and Gallagher's oxytocic preparation contains much less arginine than their pressor fraction, the information

in Table VII gives no indication as to what parts of the molecules are necessary for their pharmacological activity. Attempts have been made to throw light on this question by studying the action of enzymes and various chemical agents on the purified preparations, and this work is fully reported by Irving (110).

Enzymatic studies indicate that prolinase and arginase do not affect the activity of the oxytocic principle (79) but tyrosinase causes complete inactivation. This suggests that the tyrosinephenolic grouping is essential to the oxytocic activity. Trypsin rapidly destroys pressor and oxytocic

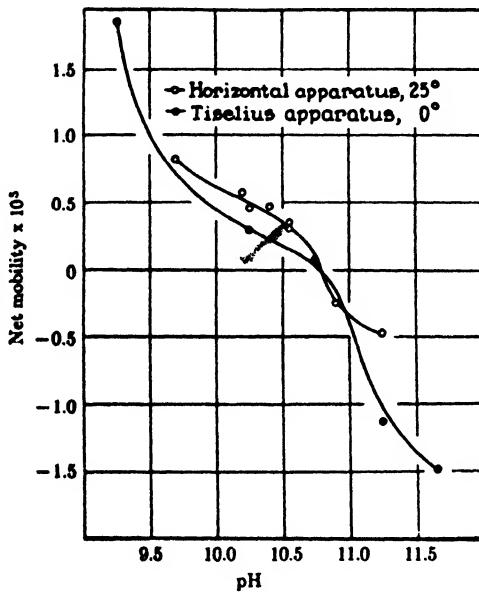


FIG. 10.—Electrophoretic mobility of pressor principle (113).

activities, whereas pepsin has no effect on either of them ("B," p. 508, behaves similarly).

Work on the effects of various chemical treatments upon the purified preparations has yielded very little information as to the structure of the active principles.

Electrophoretic studies have yielded useful information concerning the electrochemical nature of the principles. Kamm *et al.* concluded from chemical studies that both active principles were basic in character. The electrodialysis studies of Freeman (52) *et al.* and Das *et al.* (26) upon purified oxytocic preparations substantiated this view. Migration toward the cathode was readily obtained under certain conditions but migration toward the anode did not occur. They concluded from this that the

oxytocic principle was either a base or was adsorbed on basic material. Cohn *et al.* (19), however, observed that neither "principle" was able to enter a compartment where the pH was maintained at 12. Their results (Fig. 10) show that the pressor principle is definitely amphoteric with an isoelectric point at about pH 10.8. Preliminary work also indicates that the oxytocic principle is amphoteric and has an isoelectric point at about pH 8.5.

It seems likely, therefore, that the specific activities of the posterior lobe are due to closely related polypeptides of molecular weight about 2000, and as yet it is impossible to decide whether or not the physiological product of the neural lobe is a single protein molecule containing these polypeptides.

#### D. ONE AND MULTI-HORMONE HYPOTHESES

Two views are current about the chemical basis of the various pharmacological actions of whole posterior-lobe extract. One, championed originally by Abel (1), is that there exists in the gland one substance with multiple activities; the other view is that there are at least two excitants. Evidence on this issue is conflicting. The available data fall into three categories: (1) processing of extracts, (2) stimulation of gland secretion in intact animals, and (3) phyletic distribution of posterior-lobe properties.

##### 1. Processing of Glandular Extracts

Early attempts at purifying extract, summarized by Stehle (191), were based on the hope that a single physiologically active substance similar to adrenaline would be isolated from the gland. These attempts were abortive. With few exceptions, most recent work has been directed toward obtaining potent products of maximum purity to provide material for chemical analysis and to provide products with a minimum of extraneous inert matter for clinical use. Most procedures used have produced extracts with either a high or a low pressor-oxytocic ratio. Dudley (35), Schlapp (180), and Draper (31) showed that butyl alcohol extraction of acid posterior-lobe extracts removes the oxytocic property more rapidly than the pressor. Kamm *et al.* (115) and Stehle and Fraser (194), by fractional precipitation from organic solvents, obtained products with mainly pressor or oxytocic properties each contaminated with only about 5% of the other. Several workers have used adsorption (chromatographic) methods of purifying extracts (163) and have found that pressor is adsorbed more readily than oxytocic activity. The ease with which pressor and oxytocic properties can be separated *in vitro* has influenced many opinions, either implicitly or explicitly, toward the multihormone view. On the other hand, Abel consistently upheld the belief that the activities of the posterior lobe were properties of a large labile "mother molecule." Between 1917 and 1930 he and his col-

laborators described procedures by which much inert protein was removed from crude glandular extracts. He explicitly claimed that the resulting purified products exhibited pressor and oxytocic properties in the same ratio as in the original powder (page 432) and he implied that melanophore-expanding and antidiuretic activities were present in the same ratio also.

As a result of these findings Abel postulated that the gland produced one molecule with multiple activities. He recognized that extracts with varying proportions of pressor and oxytocic activity could be produced and attributed this to hydrolysis of the original parent molecule. Stehle (191) has subjected Abel's claims to penetrating analysis and has shown them to be ill-founded. Here it is sufficient to note the following: (1) Abel's procedures were described with insufficient detail to permit repetition, (2) he did not publish sufficient parallel assays to justify his contentions, and (3) some of the assay records he did publish are open to technical objection. There is no doubt that the published factual basis of his claims was unsound, but of course his hypothesis was not necessarily incorrect. Evidently Abel entertained the hope that he would eventually isolate a pure hormone, and with his well known genius for manipulative procedures this was a reasonable expectation. His incompletely described procedures were probably caused by his haste toward that goal. Later, in 1942, van Dyke (207) isolated a protein that appears to be homogeneous and has pressor, oxytocic, and antidiuretic properties in the same ratio as in I.S.P. Insufficient time has elapsed for a full appraisal of the significance of van Dyke's work, but it seems to us that if his protein is pure he has provided powerful support for the belief that the gland manufactures a single large molecule (page 456). His work has been criticized on the grounds that his tests for homogeneity were incomplete (113) and we have heard verbal criticism on the basis of the supposed low potency of his product. The former is a straight technical issue (page 456) but the latter involves an important theoretical consideration. Either pituitary hormones are themselves proteins or are attached to proteins; if the latter is true we are entirely ignorant of the chemical nature of the excitant itself. If the protein(s) resulting from van Dyke's procedures and those from separation procedures are the *actual excitants*, there is little weight in the criticism that van Dyke's product has low potency on a weight basis since the separated pressor and oxytocic products could be viewed as smaller polypeptides split off by hydrolysis.<sup>2</sup> On

<sup>2</sup> To us, on the facts now available, van Dyke's product does not appear to be of low potency. Thus a protein of mol. wt. 30,000 (p. 456) and potency  $8.5 \times$  I.S.P. might contain only one pressor polypeptide plus one oxytocic polypeptide per molecule. Then the greatest theoretical potency of either fractionated activity of mol. wt. 600 (the lowest yet suggested) would be  $(8.5 \times 30,000)/600 = 425 \times$  I.S.P. (i.e., 850 units/mg.); this potency has not yet been attained.

the other hand if van Dyke's end product has *very* low potency on a weight basis it may be suspected that the protein described is merely a carrier for the various activities.

Abel's emphasis on the importance of avoiding procedures liable to cause hydrolysis of large protein molecules is a significant issue in experiments relevant to the one *vs.* multi-hormone hypothesis controversy, because standard procedure for initial extraction involves boiling water bath treatment of powder with dilute acid or saline, and most investigators have used heat in their initial extraction. This is likely to cause hydrolysis. Ultracentrifugation studies (173) of chilled press juice also support the view that oxytocic and pressor activities are linked together in a single molecule and may be liberated by heating at about pH 4.0. Abel and van Dyke both made initial extraction in the cold, although Stehle (191) has pointed out that some of Abel's own methods might cause hydrolysis. We are impressed by the importance of hydrolysis in this connection because of our own results obtained with the use of kaolin and charcoal as adsorbents at various pH values and after various initial extractions of the original glandular material. Good separation of pressor and oxytocic activities is obtained if the initial extraction is made with *hot* water (or acid) from a high-grade, acetone-dried powder. After *cold* water (or acid) extraction of the same powder both pressor and oxytocic properties are adsorbed at equal rate and no separation can be effected. On the other hand both cold and hot extractions of several samples of ordinary commercial powder (assaying about 50% standard) have yielded good separation. It would seem from this that without hydrolysis carbon attracts both properties equally and we might plausibly postulate that they were still linked to their parent molecule. Potts and Gallagher (163, 164) using artificial zeolites as adsorbents have obtained much better separation than we have. According to van Dyke (207) their separation was accomplished without previous hydrolysis on the assumption that the initial powder used by these workers was unhydrolyzed. To bring their findings into line with our own we should have to postulate that their adsorbent had no attraction for the parent molecule but was sufficiently strong to withdraw the pressor activity from the parent. Obviously before any confidence could be established in this formulation, parallel experiments with charcoal and zeolites must be performed using the same initial extracts.

Other procedures which promise relevant information, such as ultracentrifuging, electrophoresis, and resistance to various *in vitro* changes, do not point uniformly in one direction. Data from ultracentrifugal experiments (84, 173) indicate that pressor, oxytocic, and antidiuretic activities in juice pressed from fresh posterior lobes may be associated with a rapidly sedimenting substance, presumably a protein, which is not present in fraction-

ated extracts. Electrophoresis experiments (111, 211) have shown that the mobility of pressor activity is greater than that of oxytocic, both in solutions of purified fractions and in the untreated press juice from fresh posterior lobes. Cohn *et al.* (19) in 1941 have also claimed that the substances responsible for the pressor and oxytocic activities possess different isoelectric points. Electrophoresis studies of treated extracts are of no significance in this connection, because, as we have shown with "B", subjecting the activity to various extraction procedures vastly alters its behavior in this respect. The same criticism is valid with regard to information on isoelectric points. On the other hand the difference shown by pressor and oxytocic properties in pressed juice may be good evidence for the existence of two active proteins in the juice. Various claims (57, 68) have been made that heat treatment at different pH values destroys either pressor or oxytocic properties more rapidly, but such experiments might easily hydrolyze the extracts. Rates of destruction of the oxytocic and pressor activities of commercial extracts at 99°C. were compared at various pH values. The oxytocic activity was more stable from pH 2.0–4.5 but less stable in strong acids or alkalis, *e.g.*, at pH 3.0, the point of maximum stability of both; 270 minutes heating at 99°C. resulted in 85% destruction of pressor and 34% destruction of oxytocic activity (57, 66, 90). A claim (90) to have shown a difference in heat resistance between pressor and antidiuretic activity has been shown to be invalid, for reasons of assay (50). All evidence at present indicates that pressor and antidiuretic potencies always run parallel and we are justified in believing that these two responses are caused by the same substance.

## 2. *Stimulation of Gland Secretion in Intact Animals*

If the gland initially manufactures more than one excitant we should expect that it might be feasible to withdraw one, leaving the other *in situ*. Several methods of stimulating secretion are known and of these we used injection of hypertonic sodium chloride, dehydration, and vagal stimulation. After the appropriate treatment, the glands were assayed separately for pressor and oxytocic properties. It would have been better to assay the activities in circulation but this is not technically possible. All procedures caused a reduction of residual activity in the gland, but in all cases the ratio of pressor to oxytocic activity was the same as in the controls.

## 3. *Phyletic Distribution*

International Standard posterior-lobe powder is prepared from dried ox posterior lobe, which by definition contains 1 unit oxytocic activity, 1 unit pressor activity, and 1 unit antidiuretic activity/0.5 mg. If the assays of various glands showed that some animals had pressor and oxytocic proper-

ties in different proportions, or more convincingly still in varying and irregular proportions, we should have strong grounds for suspecting there were separate entities in the gland. Not enough work has been done on these lines, but now that there is available a pressor assay (page 434) sufficiently sensitive to permit duplicate pressor and oxytocic assays on one small gland, we can expect more. Of glands so far assayed the majority have pressor and oxytocic properties in the same ratio as in I.S.P. Anomalies recorded by others are: sperm whale (59) (pressor 1 to oxytocic 0.08), finback whale (59) (pressor 1 to oxytocic 0.4), armadillo (155) (pressor 1 to oxytocic 0.1). We have assayed glands from rats, horse, sheep, pig, cat, wallaby, guinea pig, and rabbit. The first five showed a pressor-oxytocic ratio of 1 to 1, the solitary wallaby gland assayed 3 to 1, and the guinea pigs and rabbits were variable. The rabbits were of mixed breed and assayed either 1, 2, or 3 to 1. The guinea pigs of an inbred albino stock we maintain in the Aberdeen laboratories always assayed 1 to 1, but *some* glands from a heterogeneous stock assayed 2 or 3 to 1. Before drawing conclusions from these figures we should consider whether the disparities are artifacts of procedure. In an orthodox uterus assay an oxytocic titer higher than the pressor is always suspect because of histamine complication, but in all the anomalies recorded pressor is high and so this source of error is ruled out. Because of the inevitable delay in its removal, the whale gland figures would be suspect if there were evidence of differential autolysis; in fact what evidence we have indicates that the two properties are destroyed at a similar rate. In our own experiments age, sex, diet, lighting, method of killing, and treatment of glands were controlled. Hence it appears that the figures must be taken at face value. The finding of a 1 to 1 ratio generally in rodents, with an occasional 2 or 3 to 1 in some members of the same species, means either some change in internal or external environment can upset the ratio, or that there is an inherited difference. Attempts to follow up both propositions were abortive but are being continued. This information from phylogenetic studies implies either that separate excitants are manufactured by the gland *or* that the one substance is not the same in all vertebrates and can even vary among individuals of the same species. The only way to attach any meaning to the latter in view of the fact that the ratios always approximate to whole numbers, is to suppose that guinea pigs, for example, have a parent molecule with subsidiary chains, one or two of which may be absent. At present this is purely speculative.

The evidence vis-à-vis the one *vs.* multi-hormone controversy is contradictory, but a clearer formulation of the problem is now possible. At the outset we can say that one hormone in this context can only refer to a product of the *pars nervosa*, and not, as Abel implied, to the whole posterior lobe, since there is convincing evidence that "B" hormone is produced as an

independent entity by the intermediate lobe, or its homolog. Further, it is now becoming more likely that we have to consider only the question of one or two hormones from the *pars nervosa* since all pharmacological actions (with the possible exception of the water balance effect (113) in amphibia) appear to run parallel to either pressor or oxytocic properties. The strongest evidence for the manufacture of separate chemical entities by the gland is provided by electrophoresis studies on pressed juice and the results of phylogenetic studies. On the other hand all other experimental evidence is at present more consistent with the idea of a loose bond between properties, *i.e.*, a large parent molecule as envisaged by Abel. To make such a conception consistent with recent information we should have to postulate that it has several active side chains (four in guinea pig?) which can be split off readily (and remain active) by *in vitro* hydrolysis.

With improvement of technique for the assay of small quantities, we can look forward confidently to deciding whether pressor (or oxytocic) activity *alone* can be released into the circulation. If separate release does prove to occur, it will of course find a ready explanation in terms of the two-hormone hypothesis, but it will also be consistent with the conception of a large parent molecule. For, we can readily imagine either (*a*) release of the whole molecule with perhaps dissociation of the relevant activity at the appropriate effector, or (*b*) separation and release into circulation of separated activities under appropriate stimulation. Possible means for attaining either *a* or *b* are hydrolytic enzymes or adsorption to a smaller (*cf.* carbon) or greater extent (*cf.* zeolites). A final decision will depend on the isolation of an undoubtedly pure substance with multiple activities on a weight basis consistent with the known quantities stored in the gland. Until the question is settled we deprecate the unqualified use of the terms oxytocic (or pressor) principle, hormone, etc. both of which imply that pressor and oxytocic manifestations can be attributed to separate chemical entities *in the gland*. All we can legitimately say is that posterior-lobe extracts have pressor and oxytocic properties or activities. A general adoption of the term property or activity in this context begs no questions with regard to the ultimate chemical nature of the excitant or excitants.

### E. PHYSIOLOGY

The long list of pharmacological properties of posterior-lobe extracts encourages the belief that a number will prove to have a physiological significance in the intact animal. Since one of the most spectacular pharmacological effects is that on blood pressure, it was for a time thought likely that the pituitary was implicated in the maintenance of vascular tone, and hence of blood pressure. More recent work on the effect of intravenous injections into unanesthetized animals does not encourage this belief, nor

do we know of any reliable measurements of blood pressure made after removal of the posterior lobe alone which would support it. Measurements after total hypophysectomy are doubtful evidence because of the lowered metabolic rate after anterior-lobe removal and because the existence of a specific blood pressure principle from the anterior lobe has been suggested (87). All we know certainly regarding vascular tone is that frog capillaries are undoubtedly dilated after pituitary removal, and can then be constricted by injection of small amounts of pituitary extract (120). Cutaneous vasodilation does not necessarily imply reduced blood pressure. Some workers, however, believe that the posterior lobe may be concerned in the etiology of hypertension in man. Byrom (16) observed that estrogen sensitized the rat to the pressor fraction so far as the production of eclampsia-like changes in the kidney are concerned, and believed that this strengthens the view that the gland is etiologically important in eclampsia. In some cases of hypertension a proliferation of the basophilic cells of the neural lobe is found, but similar histological findings occur in the absence of abnormally high blood pressures. The physiological significance of the pressor activity remains obscure and its role as a hormone has yet to be established.

At present, we only have (1) suggestive but somewhat contradictory reports on the role of posterior-lobe extract in uterine motility at parturition, (2) suggestive reports on its role in control of gastric secretion, and (3) evidence definitely implicating it in the control of water and salt metabolism in mammals and amphibia.

(1) There is no general agreement about the significance of the *pars nervosa* in normal parturition. Pituitary extracts stimulate some parts of the human uterus more than others. Newton (152) showed that the cervix of the uterus does not respond even to high concentrations and suggests that the induced type of contraction involving only the fundus would be efficacious at parturition. The observation that uterine strips from mice and rabbits are less sensitive (170) to posterior-lobe extracts in midpregnancy than at term provides a rational basis for the onset of parturition without a raised blood content of posterior-lobe extract, as follows: During early pregnancy when the progesterone content of the blood is high, reactivity to posterior-lobe extracts is low. Toward term, when the luteal content is decreasing relative to the estrogen content, reactivity to posterior-lobe extracts increases to a maximum, culminating in expulsion of the fetus (169). The crucial experiment, removal of the posterior lobe, has not led to uniform results. The following selection from the literature emphasizes the apparently contradictory nature of the evidence. Allan and Wiles (3) found that after total hypophysectomy of cats there was quite normal delivery 11 days after the operation. Robson (171) also found that total

hypophysectomy of the rabbit did not interfere with normal birth. Selye *et al.* (183) completely hypophysectomized ten rats during the second half of pregnancy; all delivered normal litters at term. Smith (186) removed only the posterior lobe from rats and observed normal parturition. On the other hand Pencharz and Long (161) found that prolongation of pregnancy and death of the mothers before parturition sometimes followed total hypophysectomy of rats. Fisher *et al.* (39) found that cats in which total degeneration of the neurohypophysis had been caused by hypothalamic lesions delivered their young only with difficulty. In a small number of cases in which accidental mating was permitted in our stock of rats rendered insipid by hypothalamic lesions, parturition involved death of both mother and fetuses.

For the present it seems to us the difficulties in reconciling these apparently conflicting data can be resolved as follows: First we can exclude those cases in which total hypophysectomy interferes with birth on the grounds that removal of the anterior pituitary has caused diverse ancillary disturbances. We are then faced with the straight issue of why surgical removal of the posterior lobe (or whole pituitary) does not interfere with birth, while interruption of the hypothalamic tract does. So posed, we immediately suspect incomplete removal with extirpation technique (see page 475), which may be at two levels of complexity. Fisher *et al.* (39) have emphasized that in the rat surgical removal of the posterior lobe alone inevitably leaves its anterior part *in situ*, so, in the absence of evidence to the contrary, we may assume some residual posterior-lobe secretion in circulation. When the whole pituitary is removed this objection does not hold except perhaps in the rabbit, where the operation is notoriously difficult. After total surgical removal the only parts remaining of the neurohypophysis (*pars nervosa* plus pituitary stalk plus adjacent parts of tuber cinereum) are the hypothalamic nuclei and possibly part of the stalk. The question of whether the hypothalamic nuclei secrete activities similar to the *pars nervosa* is a perennial one. A number of investigators have reported excitant activity in extracts of the hypothalamus from intact animals, and O'Connor (153) has estimated from antidiuretic assays that this at the most represents only 2% of that in the gland itself. Without collateral evidence we are, of course, not justified in assuming that the hypothalamus manufactures, as distinct from stores, the excitant, although histological evidence is not adverse to the proposition that it actually secretes. It would be useful to have confirmation of the claim that the hypothalamus contains more antidiuretic activity after surgical hypophysectomy (201). This would however, not necessarily provide conclusive evidence, for a variety of reasons. One of these has not been sufficiently emphasized in endocrine literature, *viz.*, that broadly speaking endocrine glands fall into two cate-

gories: those that store considerable quantities, e.g., the *pars intermedia*, and those like the adrenal cortex (214) that secrete enormous quantities compared to their storage content at any particular moment. According to the evidence set out by O'Connor (153) and by Verney (210) we are entitled to postulate that after surgical removal of the posterior lobe significant amounts of antidiuretic activity still circulate. So we can resolve differences between results from surgical removal and hypothalamic lesions in this connection by a parallel assumption. The only doubt that arises is that the amount circulating after surgical removal must be very small, and *in vivo* experiments (anesthetized animals) indicate that fairly large concentrations are needed to activate the uterus (33).

(2) Many reports have been published that posterior-like extracts diminish gastric secretion (34) though they produce no essential change in gastric secretory response to a meal (6). Various studies, however, indicate that the effect is largely or entirely due to the vasoconstrictor action on the blood vessels supplying the glandular cells (23,125). In dogs, histamine produced a greater effect on gastric secretion when the posterior lobe was removed (143) and Cutting *et al.* (23) suggested that the neural lobe produces some substance essential for the normal regulation of gastric secretion. Further work, however, must be done before we can confidently ascribe this function to the neural lobe.

(3) There is convincing evidence that the neurohypophysis plays an important part in the control of water balance in intact mammals, and suggestive evidence that it does so in amphibia. In mammals there is a balance between the intake of water and secretion of urine. In conditions of low intake, and in certain conditions to be specified with high water load, urine is reduced in volume and is more concentrated.

Evidence for the physiological function of the neural lobe in the control of water balance can be dealt with under six heads:

- (1) Pathology of cases of diabetes insipidus in man.
- (2) Effect of surgical removal of all or part of the pituitary from animals.
- (3) Effect of secretion of hypophyseal stalk.
- (4) Experiments on isolated kidney.
- (5) Studies on hydration and dehydration of intact animals.
- (6) Studies on amphibia.

### *1. Diabetes Insipidus in Man*

Diabetes insipidus in man is characterized by dysfunction of the water balance. Large volumes of urine of low specific gravity are excreted (polyuria) and equally large volumes of fluid are drunk to compensate for this loss (polydipsia). Fluid excretion and intake can be reduced to normal by repeated injections of posterior-lobe extracts (215), though a diuresis pro-

duced in intact mammals by excessive salt intake is not controlled by these injections.

Von Hann (216) investigated the pathology of diabetes insipidus in several human cases and concluded that it can occur only if (a) the *pars nervosa* is destroyed or damaged, (b) the *pars glandularis* is functionally active (*vide infra*), and (c) there is no serious disorder of the heart and kidneys. These observations indicated that the cause of the disease was a diminished function of the posterior lobe due to lesions in the pituitary, and further investigations were carried out on experimental animals after operative interference to the pituitary region.

### *2. Effect of Surgical Removal of All or Part of the Pituitary Gland*

*a. Complete Hypophysectomy.* The main reasons for confusion in the literature can be attributed to the difficulty of operative techniques and the lack of postexperimental microscopic examination of the operation site. Hypophysectomy is seldom complete; usually the *pars tuberalis* remains attached to the hypothalamus and sometimes part of the anterior lobe is left behind. Operations of this kind were responsible for earlier claims that completely hypophysectomized animals exhibit polyuria. It is now accepted that such animals do not exhibit an abnormal urine secretion, and that a functional anterior lobe is necessary for the condition to develop fully (see p. 473).

*b. Removal of Pars Nervosa.* Initially, most of the operations on neural-lobe removal consisted of the removal of the *pars nervosa* and *pars intermedia* alone. It is difficult to remove these parts without damaging the *pars glandularis*. If, however, the infundibular stalk and median eminence (see Fig. 11) remain attached to the hypothalamus, sufficient neural hypophyseal tissue remains to regulate urine flow within normal limits. Hence, operations to remove the *pars nervosa* do not always yield animals with uncontrolled water diuresis. Usually, only a temporary increase in fluid exchange follows the operation, but permanent diabetes insipidus has resulted in some instances when the posterior-lobe stalk and part of the tuber cinereum were removed. Diabetes insipidus also does not develop in such operated animals unless the anterior lobe is functionally active. Animals made polyuric by the operation are cured by subsequent total hypophysectomy, or by continual injection of neural-lobe extracts (153).

### *3. Effect of Section of Hypophyseal Stalk and Similar Operations*

Diabetes insipidus and complete atrophy of the neurohypophysis is found to follow complete section of the supraopticohypophyseal nerve tract. This method of neurohypophysectomy has enabled much of the confusion to be cleared up. Complete section of the nerve tract is difficult

by the oral or cervical approach and it has been demonstrated that failure to interrupt even 15% of the relevant fibers will prevent the development of diabetes insipidus.

Fisher *et al.* (39) perfected a technique for cauterizing the hypothalamic regions just posterior to the optic chiasma, on either side of the third ventricle. They used a frontal approach with a Horsley-Clarke stereotactic instrument through the cerebral hemispheres and thalamus and completely severed the optic tract (Fig. 11). This operation has proved highly successful in producing diabetes insipidus in over eighty cats. Subsequent histological examination of serial sections of hypothalamus and pituitary dem-

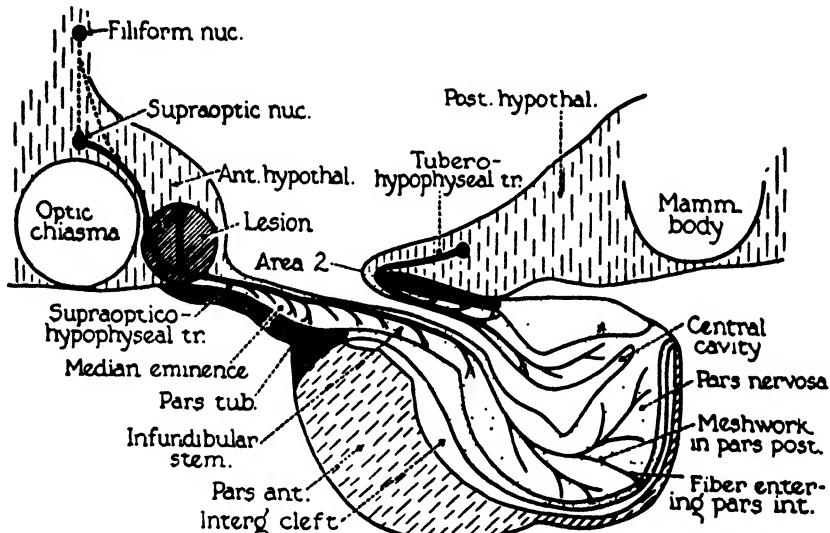


FIG. 11.—Diagrammatic representation of a medial sagittal section through the hypothalamus and hypophysis in the cat (39).

onstrate that only in those in which the supraoptic nerve tract has been destroyed, resulting in degeneration of the supraoptic nucleus, median eminence, infundibular stalk, and pars nervosa, (as evinced by lack of pituicytes in these areas), does diabetes insipidus develop.

Complete neurohypophysectomy has been performed in this way with subsequent degeneration of all neural-lobe tissue in the cat, monkey, dog, and rat. All these exhibit similar effects and phases in water metabolism imbalance which are not altered by denervation of the kidney and which can best be described under the following headings: (a) fluid exchange, (b) histology of the neural lobe, and (c) hormonal content of the neural lobe after section of the tract.

(a) After the operation there is (39,153) an immediate rapid increase in the amount of urine excreted, with a corresponding decrease in its specific gravity. In the cat this reaches its peak of 500 ml. daily by the second or third day, representing a fivefold increase over normal excretion. The daily urine output then gradually subsides, reaching normal by the sixth post-operative day. This is known as the *transient phase* of diabetes insipidus, and is also manifest in dogs, monkeys, and rats. It does not appear in all animals which do eventually develop diabetes insipidus. This transient phase is followed by a *normal interphase* lasting from 4-14 days in the vari-

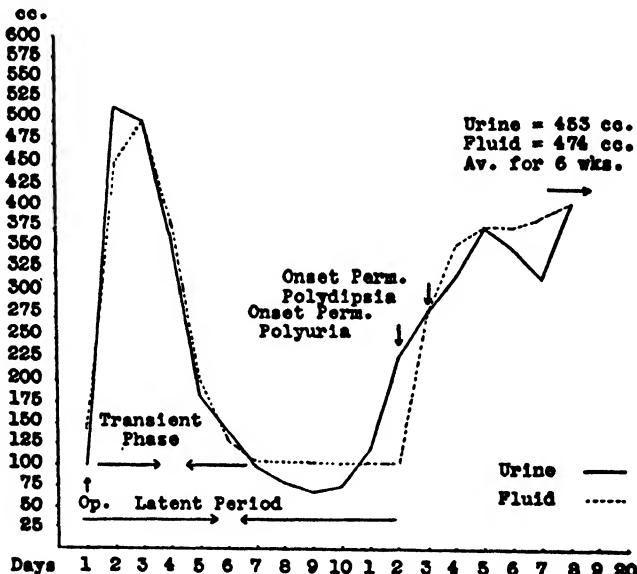


FIG. 12.—Course of polyuria and polydipsia of a typical case of experimental diabetes insipidus in the cat (39). Note that onset of permanent phase of the polyuria occurred a day before onset of polydipsia.

ous genera investigated, in which the urine output and specific gravity remains within normal levels. This in turn is followed by a sudden and marked increase in urine output, 2.5-6 times normal, with a corresponding fall in specific gravity. The urine output remains constant for long periods of time and there is every reason to believe that diabetes insipidus would persist indefinitely as it does in human cases, if the animals were kept longer. The longest-lasting experimentally produced case of diabetes insipidus has been 4 years in the dog. This is known as the *permanent phase* of diabetes insipidus. The increased urine output during both the transient and permanent phase of diabetes insipidus is compensated by an increased intake of fluid. The effects of the operation on the fluid exchange of the cat are

shown graphically in Fig. 12. It is interesting to note that, at the start of both the transient and permanent polyuric periods, the sudden increase in urine output precedes by some 12 hours an increase in the fluid intake. These and the following experiments have convinced workers that polyuria precedes and is (169) the cause of polydipsia: (i) If operated animals in permanent phase of polyuria are totally deprived of water they continue to secrete some urine. They develop a large negative water balance, as shown by the fact that they lose weight, which is made up within a few hours of their being given water. (ii) Insipid animals in which the ureters have been ligated do not drink more water than normal animals for the first few hours after ligation. They then drink more than normals due to the increased concentration of body fluids.

(b) Some attempt has been made by various workers to correlate the different phases of diabetes insipidus after section of the supraopticohypophyseal nerve tract with the histological condition of the neural division and the supraoptic nuclei. In general, operated animals show a reduction of pituicytes in these parts 8 weeks after operation. Fisher *et al.* (39) and Heinbecker and White (88) claim there is a correlation between the degree of degeneration in the supraoptic nucleus and the neural division and the degree of increase of daily urine output, partial degeneration at 10-16 days after operation accounting for the onset of the permanent polyuria. The sudden onset of the permanent phase may be due to the fact that the normal neural lobe has a large store of antidiuretic hormone. This may allow considerable and rapid degeneration of neural tissue before the urine secretion becomes affected. This threshold may be very sharp, and small differences in amount of hormone available may markedly affect the urine secretion. In this connection Heinbecker and White (88) have shown that failure to interrupt the fibers of as few as 15% of cells innervating the neural division will prevent the development of any permanent polyuria, while O'Connor (153), working on the antidiuretic effect of faradic stimulation of normal and neurohypophysectomized dogs, estimated that from 2-7% of the normal neural-lobe activity is secreted after neurohypophysectomy. Thus it is usual in animals with diabetes insipidus to find more than 5% and less than 15% of normal secretion. The cause of the increased urine output immediately after operation is unknown. It is not due to degeneration of the neural lobe, because it is absent in some operated animals which later develop a permanent polyuria, and because it is followed by a normal interphase during which neural lobe secretion must be fairly normal.

(c) The antidiuretic potency of the posterior pituitary of animals at various intervals following pituitary stalk section have been investigated by numerous workers. In all cases of permanent polyuria the posterior

lobe contains about 5% of the normal amount of antidiuretic activity. The hormonal content of the neural lobe is not the controlling factor in the development of the transient polyuria or the normal interphase. It has been suggested (136) that in the transient phase the polyuria is severe because the hormone fails either to be secreted or to reach the kidney tubules. This claim is supported by the fact that at this stage very small doses of Pitressin (1-5 milliunits) will suppress diuresis. In the normal interphase the hormone content of the posterior lobe falls from 100 to 10%, yet the excretion of water remains very similar to postoperative levels. Results indicate that only about 5% of the neural division is necessary to maintain normal urine secretion.

These facts provide the basis for a rational explanation of diabetes insipidus. The supraopticohypophyseal system regulates the secretion of antidiuretic hormone. Interruption of the supraopticohypophyseal tract in the hypothalamus causes atrophy of the whole neurohypophysis. The consequent deficiency in the antidiuretic hormone produced, as evinced by continued excretion of large volumes of urine, can be corrected by injection of posterior-lobe extracts. Likewise section of the stem high enough to cut all of it and the median eminence away from the hypothalamus brings about a similar atrophy and deficiency.

#### 4. *The Significance of Anterior Pituitary in Etiology of Diabetes Insipidus*

Since Von Hann's time there has been dispute about the relative importance of the anterior lobe and of the several parts of the neurohypophysis in the etiology of *diabetes insipidus*. To us the most acceptable view (39,89) is that the dysfunction results from the absence of neurohypophyseal control of urinary function, but that the presence of the anterior pituitary is necessary to maintain the normal trophic state of the body which makes the increased water discharge obvious. Thus full manifestation of diabetes insipidus is dependent on complete absence of the neurohypophysis and on the presence of a functional anterior pituitary. A partial diabetes insipidus can result if traces of the neurohypophysis remain, provided the anterior pituitary is functional; and this partial condition may also obtain temporarily after removal of the whole pituitary until the general trophic condition of the body degenerates due to the absence of the anterior pituitary. There is meanwhile no acceptable evidence that the anterior pituitary exerts a specific influence, either directly or indirectly. At this juncture it is convenient to draw attention to the fact that after surgical removal of the neural lobe there is sufficient neural tissue remaining to prevent *diabetes insipidus*, but not sufficient to enable the animal to conserve high water load in emotional stress (*vide infra*).

### 5. Experiments on Perfused Kidney

Isolated kidneys, perfused with either defibrinated blood or saline, excrete part of the perfusion fluid through the glomeruli. When posterior-pituitary extract is added to the perfusion solution the excretion is never so great and can be moderately controlled by increasing the concentration of the extract. Verney (208,209) determined the effect on urine secretion of switching various parts of the circulation of a dog into series perfusion with a dog heart-lung-kidney preparation. It was found that, when the head and neck of the donor dog were perfused in series with the isolated kidney, a marked inhibition of diuresis ensued. Similar perfusion of hind limbs was ineffective on urine secretion. Compere (20) and Brull (11) found that when the kidney of a normal dog was connected in the circulation of another normal dog it exhibited a normal urine output, and when similarly connected with the circulation of an insipid dog it showed polyuria.

### 6. Studies on Hydration and Dehydration of Intact Animals

a. *Hydrated Animals.* Verney (210a) in his Croonian lecture has analyzed the mechanism whereby external stimuli inhibit the diuresis following administration of water by stomach tube to intact animals. He showed that, if the water load is the initial stimulus to which the kidney eventually responds, there is a 15-minute lag between presentation of stimulus and maximum response. This time is not altered if the kidney is denervated and suggests a hormonal mechanism.

Muscular exercise *per se* does not inhibit the diuresis of hydrated animals, but emotional stress produces an antidiuretic response which is manifest even after denervation of the kidneys and adrenals. Changes in renal blood flow are not responsible, for injection of either adrenaline or pituitary extracts inhibits urine flow, but the response to the former is transitory, whereas the response to the latter simulates that of emotional stress (Fig. 13). After surgical removal of the posterior pituitary only about 5% of the original inhibition evoked by emotion stress remains, but the animal does not lose its ability to conserve its own tissue water, *i.e.*, does not exhibit diabetes insipidus, presumably due to the residual activity in the hypothalamus. Sometimes there is superimposed on this pituitary mechanism a more rapid antidiuretic response to emotional stress which is abolished by section of the splanchnics and the adrenal and kidney nerves.

Verney (210) has also shown that injection of adrenaline (or tyramine in equipressure doses) will stop the slow pituitary inhibition and concludes that increased cranial blood pressure will inhibit release of *pars nervosa*

excitants. This provides an explanation in part for the irregular appearance of the slow inhibition in normal dogs. Increased sympathetic activity during emotional stress might thus prevent release of the hormone (162,210). The one remaining link in this satisfactory chain of evidence, *viz.*, demonstration of increased pituitary substance in the blood, awaits a more sensitive antidiuretic assay. Summarizing, we can say that the slow inhibition of diuresis in response to emotional stimulation of hydrated dogs is dependent on an intact neurohypophysis (*i.e.*, *pars nervosa* as well as hypothalamus).

*b. Studies on Dehydration.* Dehydrated intact rats secrete urine with antidiuretic properties, whereas urine obtained under similar conditions

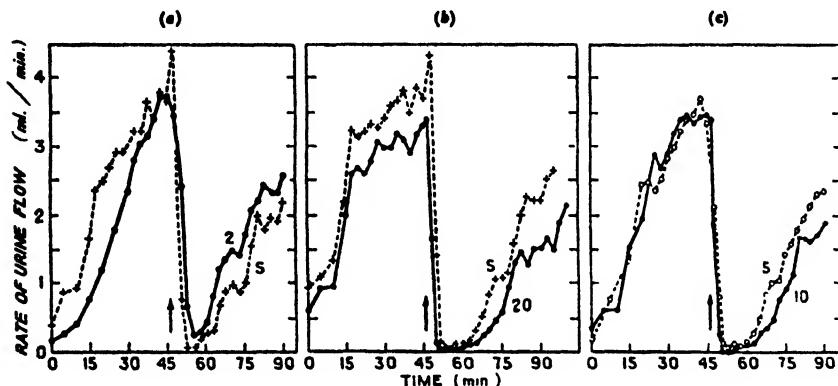


FIG. 13.—Estimation of amount of antidiuretic substance liberated in response to 44 sec. emotional stimulus before removal of posterior lobe in bitch weighing 11.2 kg. Dose of water = 300 ml. Stimulus and injection were given at the arrow: (a) inhibition produced by emotional stress (graph S) is compared with that resulting from intravenous injection of 2 milliunits (graph 2); (b) another inhibition from an equal stimulus (graph S) is compared with that from 20 milliunits (graph 20); (c) inhibitions resulting from 5 and 10 milliunits (210).

from hypophysectomized animals does not exhibit antidiuretic properties (68). From this we are led to believe that under conditions necessitating water conservation the *pars nervosa* is activated. Attempts to investigate the problem by measuring the pituitary substance in circulation have given conflicting results. No positive result is acceptable to us. The assay method would have to be both specific and exceptionally sensitive. In the hydrated dog, the intravenous injection of 1 milliunit posterior-lobe extract suffices to reduce the urine flow to the resting level. Under physiological conditions, therefore, there is no need to postulate that amounts greater than this are ever circulating in the blood stream. So the differ-

ence in the posterior-pituitary antidiuretic activity of 1 ml. blood taken from a dog at normal and at diuretic rates of urine flow need only be 0.0015 milliunit, even assuming that the hormone is restricted to the blood stream and that the volume of circulating blood in the dog is 600 ml. (153,210). Tests capable of detecting such minute differences are not available. The rat test of Burn (14) can only measure amounts greater than about 20 milliunits of antidiuretic activity, while 0.1 milliunit is the smallest dose consistently detectable in the dog. These tests are not specific to pituitary antidiuretic activity; other substances capable of inhibiting water diuresis may occur in extracts of blood. For example, Molitor and Pick (145) showed that histamine may cause antidiuresis, and Theobald and White (200) observed another antidiuretic substance in liver extracts. In the absence of demonstration of its source, the antidiuretic activity of blood or cerebrospinal fluid described by Marx (132), Marx and Schneider (133), and Melville (138) and corresponding to 5-10 milliunits pituitary antidiuretic substance/100 cc. cannot be accepted as of pituitary origin. Gilman and Kidd (69) and Walker (217) also found antidiuretic activity in blood samples but were unable to establish its pituitary origin.

Since dehydration tends to increase the osmotic pressure of blood plasma, experiments have been carried out on the effect produced by increasing the osmotic pressure by other means. Injections of sodium chloride both deplete the stored activity of the *pars nervosa* of rats and cause inhibition of diuresis in hydrated dogs. Verney (210), using a variety of substances for increasing the osmotic pressure of the plasma, has shown that the "osmoreceptors" are not uniformly sensitive to all substances injected in equi-pressure amounts. Since preliminary experiments indicate that ligation of the internal carotids interferes with the inhibitory response, it is probable that the "osmoreceptors" are located in the capillary bed of the pituitary.

Current information about the role of the *pars nervosa* in maintaining water balance of mammals can be epitomized as follows: (1) Provided the anterior lobe is functional, removal of the entire neurohypophysis results in diabetes insipidus, which is characterized by a primary polyuria and a secondary polydipsia. Removal of the *pars nervosa* alone does not cause this condition. (2) The antidiuretic response of hydrated animals to emotional stress (presumably by nervous control of the pituitary) is dependent on a completely intact neurohypophysis. (3) Dehydration (or raising the plasma osmotic pressure) evokes inhibition of diuresis mediated by increased pituitary secretion. After surgical removal of the *pars nervosa* from the dog antidiuretic response to saline is reduced to 5% of normal. Dehydration evokes no measurable release of antidiuretic substance after surgical removal of the *pars nervosa* (plus *pars glandularis*) in rats.

### 7. Studies on Amphibia

The foregoing information is all concerned with water balance in mammals. When describing the pharmacological action of posterior-lobe extracts (page 451) an analysis was made of its effect on the water balance of amphibia. In amphibia we are dealing with two separate processes, *viz.*, entry of water through the skin, and renal effects comparable to those in mammals. We have now to inquire whether there is any evidence implicating the *pars nervosa* in the physiology of amphibian water metabolism, or whether the effects previously detailed are of purely pharmacological interest.

If the neurohypophysis of the intact frog exerts a renal effect similar to that in mammals we should expect removal of the anterior lobe alone or total hypophysectomy to have no effect, and hypothalamic lesions (presumably leading to total degeneration of the neurohypophysis) to cause polyuria. Experiment shows this to be so. It would be interesting to know the effect of surgical removal of the posterior lobe alone, but it is doubtful whether this has ever been accomplished without damage to the anterior lobe or hypothalamus, except in *Xenopus*, in which water relations have not been studied. So in the frog it would appear that a renal control similar to that in mammals exists, but the data are not complete (94).

A strong *prima facie* case for the involvement of the *pars nervosa* in the control of water entry through the skin of amphibia is provided by three observations: (1) Injections of posterior extracts cause less increase of body water in amphibian species from drier habitats as compared to species from wetter habitats, and have little effect on intake of water in fish which do not normally experience difficulties due to desiccation. (The skin of higher animals is impervious to water, so, although negative results have been reported from experiments on the effect of pituitary injections on intake of water, their significance seems obscure). (2) The high ratio of "water balance principle" to other neural-lobe activities in frogs as compared to members of other vertebrate classes. (3) Injection of posterior-lobe extract diminishes water loss of intact frogs in dry surroundings.

Evidence from hypophysectomy etc. is incomplete, difficult to interpret, and cannot at present be said to afford much support. We should expect difficulties of interpretation because after neurohypophysectomy a polyuria develops and presumably this will result in a large water intake through the skin (since frogs do not drink). Thus the loss of the potentiating effect of "water balance principle" on entry of water will be masked. Even so, it is surprising to find that hypothalamic lesions cause an *increase* of body water. Total hypophysectomy gives variable results and removal of the anterior lobe alone has no effect. Two other observations are at present difficult to reconcile with a simple scheme implicating the "water balance

principle" in normal physiology, *viz.*, (1) after total hypophysectomy<sup>3</sup> frogs are not more prone to water loss on desiccation, but injection of extracts does protect intact frogs from desiccation; and (2) desiccation does not decrease the amount of "water balance principle" in the pituitary. That in fact the response is a very complicated one, involving at least both lobes of the pituitary, is indicated by the observation that removal of the anterior lobe or the whole gland is followed by a marked decrease in the response to posterior-lobe extracts with respect to their effect on water intake. In summary, we can say that the presumptive evidence implicating the *pars nervosa* in the normal physiology of water intake is sufficiently strong to justify systematic efforts to clarify some of the (at present) contradictory results.

#### IV. Intermediate Lobe

##### A. PHYSIOLOGY

Of the several pharmacological actions (page 495) attributed to "B"-containing extracts, only its melanophore-excitant activity has so far been shown to have a physiological role in the intact animal (102,157,222).

Its potential function with regard to melanin synthesis and carbohydrate metabolism are at present under investigation in our laboratories.

The role of "B" hormone in chromatic physiology has been extensively investigated, but judging by summaries written by people not immediately engaged in the field it is one of the most imperfectly understood by endocrinologists in general. Since a detailed discussion of melanophore changes is included in Chapter IX, C, a brief treatment here will be sufficient.

##### 1. *The Nature of Chromatophore Activity*

A large number of cold-blooded vertebrates belonging to the classes Pisces, Amphibia, and Reptilia exhibit a striking dermal pigmentary response to changes of illumination, temperature, etc. Visible chromatic response in these forms results from the "expansion" and "contraction" of dermal and epidermal chromatophores which are distinguished as melanophores, xanthophores, or erythrophores, according to whether they contain black, yellow, or red pigment. When chromatophores are contracted the skin is pale; when expanded the skin is dark (Fig. 14). The chemistry of the various pigments has been described by Fox (43). Most of our reliable information concerns melanophores. The terms *contracted* and *expanded*, used by early workers who believed that chromatophores were ameboid, are retained in accordance with custom. An alternative view is now generally, but not universally, accepted. Conclusive evidence for it

<sup>3</sup> The key observation, *viz.*, desiccation after total neurohypophysectomy (leaving the anterior lobe intact) has apparently not been made.

in one teleost, *Fundulus*, has been furnished by observations made by Matthews (134) using a tissue culture technique. He was able to distin-

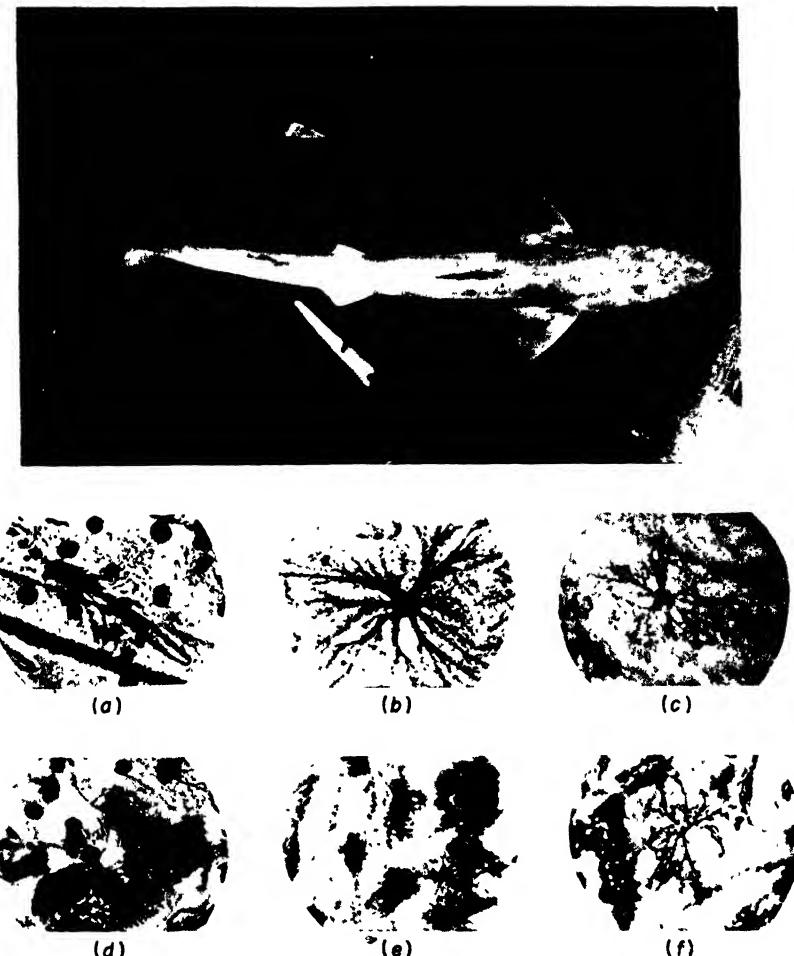


FIG. 14.—*Squalus* (dogfish), normal and hypophysectomized, on black background, and melanophores at different stages of expansion (220). (a) Dermal and epidermal melanophores fully contracted; (b) intermediate condition of dermal melanophores; (c) expanded condition of dermal melanophores; (d) intermediate condition of epidermal melanophores; (e) expanded condition of epidermal melanophores; (f) static epidermal pigment.

guish clearly a fixed cell boundary unaffected by migration of the pigment granules within it. By use of magnifications up to 1000 diameters (95) it

has been possible to discern the same structure in frog tadpoles. To describe these states more accurately the terms *dispersion* and *concentration* of pigment (melanosomes) have been used by some authors. On the other hand there is little doubt that in the early life of some species the chromatophores are ameboid and some authors (116) are loath to accept the view that in general chromatophore peripheral margins are static.

Undoubtedly a great deal of the confusion in the literature on chromatic effector systems is due to persistence of workers in describing chromatic behavior in terms of the macroscopic appearance of the animal as dark, intermediate, pale, etc. For purposes of natural history vis-à-vis the protective coloration controversy, a case might be made out for recording gross macroscopic appearance *as well as* the individual behavior of the melanophores, but for physiological studies bearing on the behavior of an individual effector organ and its co-ordination there are several valid ob-

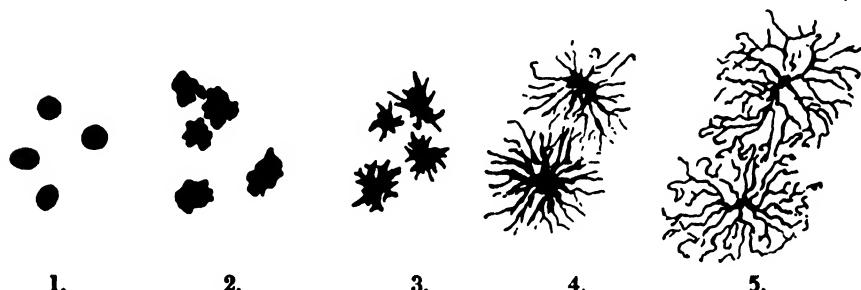


FIG. 15.—Hogben melanophore index (107).

jections to the system. The terms used are inexact. Hence the records of different workers are not comparable and communications are verbose. A more important objection, which also applies to the photoelectric method (99) is that macroscopic appearance depends on several different microscopic effects and on the previous history of the animal. The last point is of particular importance. Animals kept in conditions that maintain an expanded condition of the melanophores rapidly develop more melanin and more melanophores. Under conditions that evoke melanophore contraction the reverse happens. We have emphasized this source of error by publishing photographs (124) showing that it is possible for an animal (with many melanophores) to be darker when all its melanophores are contracted than another animal with few, but expanded, melanophores. By use of the Hogben melanophore index (Fig. 15) all these pitfalls are avoided and a precise estimation of individual effector activity is obtained. Without its use precise analyses of effector activity, and hence pituitary activity, are not obtainable.

Chromatic behavior of vertebrates may be elicited by a variety of physical agencies and the relative importance of these several agencies is not the same in different species of the same taxonomic group. Broadly speaking, however, light is the most important, and commonly predominates over all other agencies, and since in any case it gives most of the significant information with regard to pituitary activity we shall here confine our attention to it.

It is convenient to distinguish four classes of melanophore response to light (*a*) unco-ordinated nonvisual (primary) or dermal response, which is independent of the eyes, central nervous system, and pituitary, so that the melanophore almost certainly behaves as an independent effector; (*b*) a co-ordinated nonvisual response which is independent of the eyes, but involves either nervous or pituitary co-ordination between stimulus received by some other receptor and the melanophore (See Table I in Waring's review, 222); (*c*) a secondary ocular response, and (*d*) a tertiary ocular response. The response to photic stimuli with either *a* or *b* is expansion of the melanophore in light, and its contraction in darkness. The secondary ocular reaction (black-background response, *i.e.*, expanded melanophores) is evoked by transferring an animal from darkness to superior illumination in light-absorbing surroundings (*e.g.*, black tank). Light initiates this reaction when it falls on a *B* area located in the ventral part of the retina of the vertebrate eye. The tertiary ocular reaction (white-background response) is evoked by transferring animals from superior illumination in light-absorbing to superior illumination in light-scattering surroundings (*e.g.*, white tank). Light initiates this reaction when it falls on a *W* area, dorsal to the *B* area, of the retina of the vertebrate eye (104). It is generally true to say that most vertebrates that exhibit background response (*i.e.*, *c* and *d* also show *a* or *b*, and that when they do the ocular response predominates). The role of nervous co-ordination is taken up by Parker in Chapter IXC. In this account we shall confine ourselves to the ocular responses.

## 2. Secondary Ocular Response

In elasmobranchs (Fig. 14) and amphibia there is unequivocal evidence that this response is mediated by "B" hormone from the intermediate lobe of the pituitary. The chain of evidence is as follows: in complete darkness (or eyeless animals in light or dark) the m.i. (melanophore index) is intermediate (*i.e.*, ca. 3.0) and on a black background the melanophores are expanded (ca. 5.0). The speed of change (222) is consistent with hormonal co-ordination, and peripheral-nerve section has no effect. Hypophysectomized animals are permanently pale. The pituitary from animals in complete darkness (and also from illuminated animals) contains more than sufficient to darken the animal fully. Blood transfusion from

intact animals with fully expanded melanophores into pale hypophysectomized animals shows that the hormone is detectable in adequate quantities in circulation. Experiments with both perfused isolated limbs and excised skin in saline (124a) show that the effect of the excitant is direct on the melanophores. With the exception of the eel, the evidence from teleosts is not so complete but suggests that, with the possible exception of *Fundulus*, further work will show that they conform to the amphibian pattern in this respect. Reptiles so far investigated fall into two categories. There is sufficient, but incomplete, evidence to indicate that *Anolis* and *Phrynosoma* are like elasmobranchs and amphibians, but chameleons execute this change so rapidly that a significant contribution by the pituitary is not to be expected. However a final judgment awaits the effect of hypophysectomy, which has not been performed.

### 3. *Tertiary Ocular Response*

There is less unanimity about the co-ordination of this response. When amphibians and elasmobranchs are transferred from a black background to a white the melanophores very slowly contract. Peripheral-nerve section has no effect on the response, and estimation of the effective "B" content of sera from animals equilibrated on white and black backgrounds shows that the former contains no measurable amounts. So since the *B* area of the retina is constantly illuminated (and hence impulses potentiating release of "B" are continued during the change) illumination of the *W* area of the retina must release impulses into the optic nerve which do one of two things, *viz.*, inhibit the release of "B" or release another hormone ("W") with contracting (or "B"-inhibiting) properties. The implications of the two hypotheses are set out in Table IX and in Fig. 16. There are several kinds of evidence for the existence of a second, melanophore-contracting, hormone ("W") produced either by the *pars glandularis* or some other endocrine controlled by it: (a) After removal of the *pars glandularis*, leaving the posterior lobe intact, animals are permanently dark (m.i. 5) irrespective of lighting conditions. (b) After removal of the whole pituitary some animals (*e.g.*, *Xenopus* and eel) do not exhibit pallor so complete as intact animals on a white background or animals with the posterior lobe alone removed. (c) After removal of the *pars glandularis* as well as the posterior lobe animals are more sensitive to "B"-containing extracts than after removal of the posterior lobe alone (Fig. 17). (d) After removal of the whole pituitary from eels equilibrated at m.i. 5, the animals slowly settle down to m.i. 3.2, which is the equilibration figure for hypophysectomized animals (222). After removal of the pituitary from animals at m.i. 4.2 the m.i. decreases to 2.0 and then slowly rises to

3.2 (Fig. 18) (221). This can be rationally interpreted if we postulate the existence of a contracting hormone ("W") in the latter which is more slowly extinguished than "B." With regard to this, the time relations of transition for intact animals also imply that "W" (if it exists) is more slowly

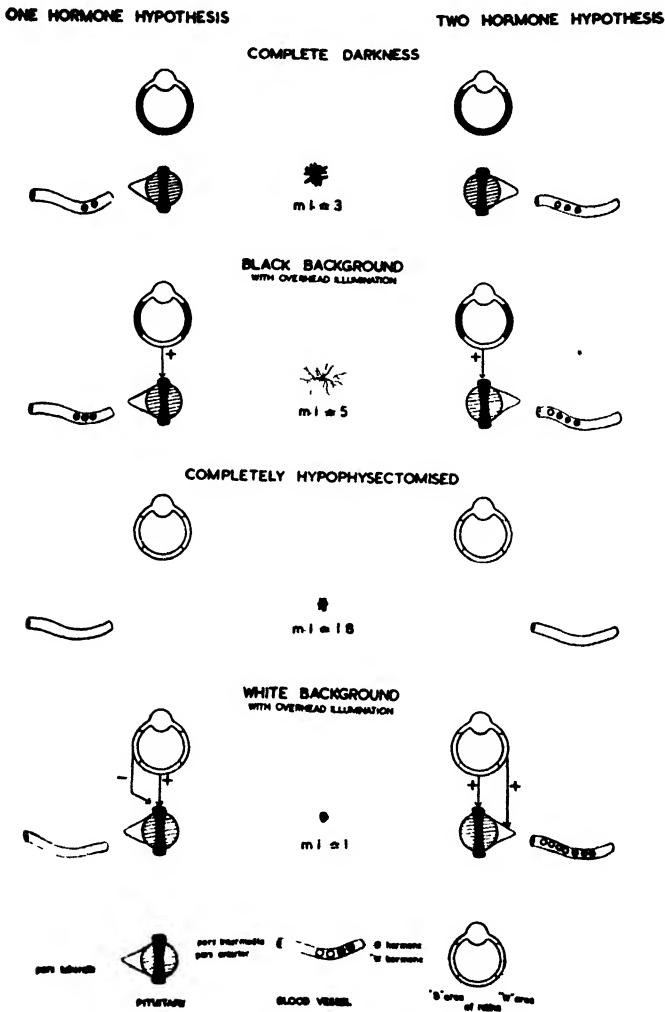


FIG. 16.—Tertiary ocular response of *Xenopus*.

excreted (148). In addition, observed time relations of intact animals in transition from one state of equilibrium to another receive more plausible explanation if we postulate the existence of two hormones. With regard to the evidence set out above, *a* and *c* would permit rational interpretation

on the basis of the second hormone's being purely inhibitive to "B" action, but both *b* and *d* demand an excitant with contracting powers acting directly on the melanophore.

The two-hormone hypothesis has not escaped criticism and the chief

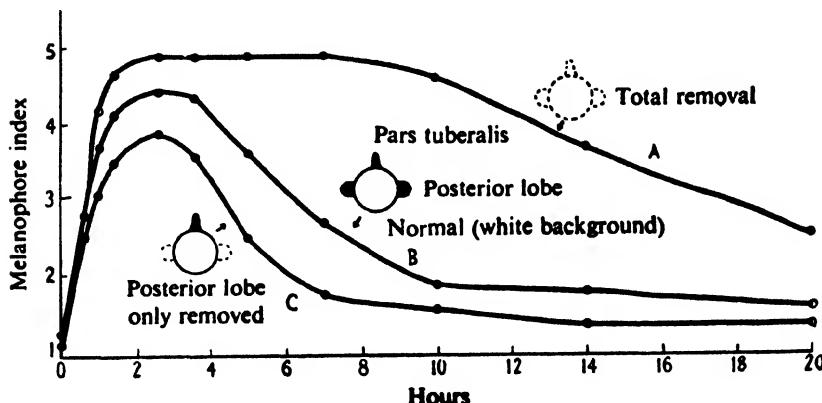


FIG. 17.—Tolerance of three series of six toads to pituitary extracts (107). Animals selected for same degree of pallor and weight ( $30 \pm 2$  g.). Each received 0.075 ml. 10% extract of posterior lobe deprived of all pressor and oxytocic activity by method of Dale and Dudley (25a). Temp. 14°C.

TABLE X (220)  
MELANOPHORE INDICES OF CERTAIN ANIMALS<sup>a</sup>

Condition	<i>Rana</i>			<i>Xenopus</i>			Elasmo-branchs			<i>Anguilla</i>		
	B	W	D	B	W	D	B	W	D	B	W	D
Normal.....	5.0	1.5	3.0	4.5	1.5	2.5	5.0	1.5	3.0	5.0	1.0	3.5
Whole pituitary removed....	1.0	1.0	1.0	2.0	2.0	2.5	1.0	1.0	—	3.5	2.0	—
Anterior lobe removed.....	5.0	1.5	3.0	4.5	1.5	2.0	5.0	5.0	—	5.0	5.0	—
Anterior pituitary and <i>pars tuberalis</i> removed.....	—	—	—	5.0	5.0	—	—	—	—	—	—	—
Posterior lobe removed ....	—	—	—	1.0	1.0	—	1.0	1.0	—	—	—	—

<sup>a</sup> B = overhead illumination, black background. W = overhead illumination, white background. D = complete darkness.

objections to it can be set out as follows: (1) While extracts with melanophore-contracting powers have on rare occasion been produced, these powers may have been due to traces of the reagents used. (2) The permanent darkening after removal of the *pars glandularis* has been attributed to disruption of nerve tracts connecting the hypothalamus with the inter-

mediate lobe (2). (3) It has been pointed out that the differential tolerance might also be due to the lowered metabolic rate of animals without their anterior lobe (2). There is some merit in all these criticisms, which have been discussed fully elsewhere (224), and they will no doubt form the subject for more experimental investigation. Meanwhile as a working hypothesis the two-hormone formulation is justified in that it explains all the anomalies, some of which receive no interpretation in terms of the

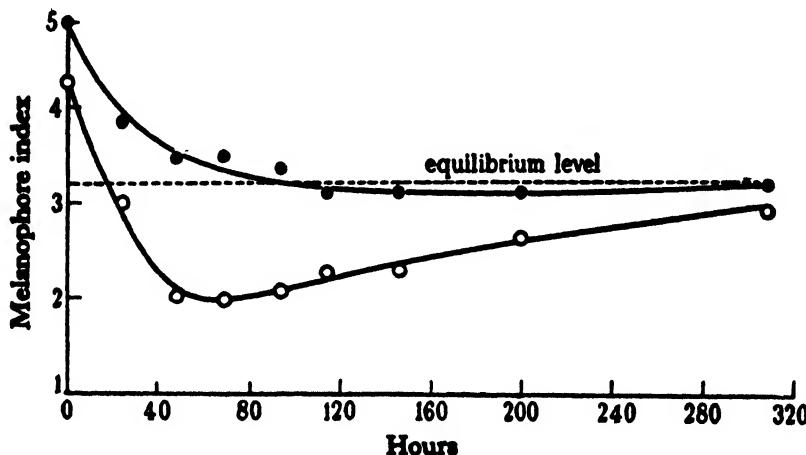


FIG. 18.—Reaction of two groups of eels after total hypophysectomy. ● = previously equilibrated at m.i. = 5. ○ = previously equilibrated at m.i. = 4.2. Black background (221).

fluctuation of one expanding hormone, while at the same time posing questions susceptible to test.

The tertiary ocular response of teleosts involves a complicated relationship between pituitary hormones and peripheral innervation, and in different species either may dominate the other. The "W" hormone mechanism (or reduced secretion of "B"), as a means of bringing about contraction of the melanophores, has been superseded by direct innervation to different degrees in different species. The result is that background reversal in some species is extremely rapid. Sufficient information is now available to build up a picture of how peripheral innervation has taken an increasingly dominant role (cf. Sect. C3 of Chapter IX). In the eel the effective co-ordinator in intact animals is the same archaic pituitary mechanism described for amphibia and elasmobranchs; but, unlike the melanophores of amphibia and elasmobranchs, those of the eel are directly innervated. The peripheral innervation takes no part in normal chromatic function because its action is completely dominated by the pituitary mechanism.

When eels are completely hypophysectomized it is still possible to discern a small measure of chromatic response and this is under the control of the peripheral nerves. In *Phoxinus* and pleuronectids the importance of direct innervation has increased so that effective control in the intact animal is nervous. But if the "B" content of the blood is artificially increased by injection into a pale fish on a white background, the animal will darken, thereby showing that although the peripheral nerves can override the amount of "B" secreted by the animal's own pituitary they cannot cope with an increased amount. In *Fundulus* peripheral innervation is completely dominant, so that injection of large doses of "B"-containing extract do not cause darkening. If, however, a small area is denervated, its melanophores will readily respond to "B" by expansion.

The aforementioned four categories of chromatic response to light are sometimes referred to collectively as "physiological" changes because they involve redistribution of pigment only. If an animal is kept under conditions that cause prolonged expansion of melanophores, there is in addition an absolute gain of melanin and melanophores (27, 197) which has been measured both by counting melanophores and by colorimetric estimation of melanin removed from the skin. The reverse holds when melanophores are maintained in a state of contraction. This is called morphological change. It is fairly evident that the two probably shade off into each other, but no confusion (158) between them is possible provided physiological changes are measured on the melanophore index. Little work has been done on the possible hormonal basis for morphological changes but our own preliminary, unpublished work indicates that completely hypophysectomized toads (*Xenopus*) either do not lose pigment or do so very slowly and that animals from which the *pars glandularis* alone has been removed gain pigment rapidly. This would seem to indicate that build-up of pigment is potentiated by the posterior lobe and breakdown by the *pars glandularis*. Work is now proceeding in our laboratories to test this proposition.

#### B. METABOLISM OF THE HORMONE

Observations on the fate of "B" in the body are for the present conveniently considered under the headings of cold-blooded animals and mammals, because parallel observations have not been made in the two groups.

##### 1. Cold-Blooded Animals

"B" can be detected in the blood of amphibia (159), elasmobranchs (219), and eel (223) equilibrated on a black background by injecting serum from them into pale animals. We have not been able to detect "B" in the urine of untreated amphibia, nor after intraperitoneal injection of

large doses of "B" extract into *Bufo* (123). It appears therefore that both endogenous and injected "B" are destroyed in the tissue. This receives support from two other sources: (1) When *Xenopus*, *Rana*, or *Anguilla* (223) with fully expanded melanophores are pithed and perfused with saline, the melanophores contract, and then expand when "B" extract is added to the perfusate. When straight saline is again perfused the melanophores do not contract. This implies one of two things: either the perfusing fluid does not permit the passage of the hormone back through the capillary walls, or the first perfusion removed from the tissues some substance which normally destroys "B". In *Anguilla* we found that the melanophores themselves were not inactivated, since adrenaline added to the perfusate promptly contracted them. (2) The melanophores in isolated strips of dogfish skin expand when placed in saline containing pituitary extract and, an hour or so later, contract. Discrimination between different doses is shown. The subsequent contraction suggests that the skin destroys the hormone. That this is so was shown by keeping samples of the pituitary extract in saline under identical conditions but without skin. When the melanophores had contracted again in the experimental series, skin placed in the control solutions showed that no measurable decrease of potency had occurred.

## 2. Mammals

Attempts to detect "B" in mammalian blood are complicated by the necessity for pretreating blood (122), which is toxic to the test animals if untreated. After various trials we adopted the procedure of letting blood directly into acetone and extracting the precipitate. In trials in which a known quantity of extract was added to blood, 60% was recovered. Using this method we have been unable to detect "B" in the blood of normal rabbits (or man) during tests in which the animals were subjected to a variety of lighting conditions. Large doses of "B"-containing extract injected subcutaneously or intramuscularly did not reach a measurable level in circulation. On the other hand after intravenous injection "B" could be detected in the circulation and serial samples showed that it was removed from circulation at about the same speed as injected pressor and oxytocic properties (122). Both Jores (114) and Levinson (128) have claimed that "B" can be detected in the blood of untreated mammals, but there is serious objection to their assay methods (123, 124). On the face of it it seems that mammals differ from pisces and amphibia in not having measurable quantities in circulation, but we are at present unable to eliminate the possibility that the differences found are due to the necessity for pretreating mammalian blood.

There have been many claims to have detected "B" in urine of man and

its variation in different circumstances has been described, *e.g.*, menses, pregnancy, retinitis pigmentosa, and other conditions in which the pituitary is believed to be involved. We have reviewed these claims elsewhere (123); all are open to criticism on the grounds of assay, and the fact that crude urine sometimes contains substances other than "B" which will evoke expansion of melanophores. Since then Mutch and McKay (147) working under our direction and using *Xenopus* for assay examined a large number of urine samples from normal (European) and from retinitis pigmentosa patients. In no case could they definitely detect "B" in the urine. There has been suggestive evidence for its existence in urine of negroes and Chinese and this is being followed up. Massive subcutaneous or intramuscular injections of "B"-containing extracts into mice led to no detectable amounts in the urine. After large doses are injected intravenously into rabbits, "B" can be detected in the urine. This finding has probably no physiological significance for another reason besides the obvious one, that the "B" content of the circulation was abnormally augmented. In different extracts "B" activity may be associated with or bound to different-sized molecules of protein hydrolyzates. This seems to be the only possible interpretation of the otherwise contradictory findings of Zondek and Krohn (228) and Dreyer and Clark (32) with regard to the relative diffusion rates of "B," pressor, and oxytocic properties through membranes. So we shall not know whether "B" as ordinarily released from the intact pituitary can pass the kidney until we know in what form "B" is normally secreted or until we can augment endogenous secretion into the circulation.

Studies on pressor and oxytocic properties showed that *in vitro* the liver is a potent detoxicator). In view of this, and the suspicion from carbohydrate studies (page 501) that "B" has a seat of action in the liver, we examined liver of untreated guinea pigs and rabbits for "B" (122). From liver we extracted a melanophore excitant which shared with pituitary extracts the ability to raise the melanophore index of hypophysectomized *Xenopus* to 5, and to be adsorbed on and eluted from charcoal. Other excitants that we have found in urine and other body fluids do neither of these things; they raise the m.i. of *intact* toads, presumably due to the initiation of endogenous secretion and they are not adsorbed on charcoal. These two criteria are as far as we can go at present in identifying the excitant substances from the liver and the pituitary as the same.

From this incomplete information we form this provisional picture: "B" released from the intermediate lobe reaches measurable dimensions (by current methods of assay) in the circulation of coldblooded animals but not of mammals. It is destroyed by the tissues and is adsorbed by the liver. It is not normally excreted by the kidney and after subcutaneous injection it does not reach a concentration in the circulation sufficient to

pass the renal threshold. After intravenous injection into mammals it can be detected in the urine. At present it appears that its chemical behavior is similar to that of pressor and oxytocic properties.

### C. PHARMACOLOGY

The following effects have been described as resulting from the injection of extracts containing melanophore-expanding hormone "B": (1) expansion of chromatophores (102,157,222); (2) increase of melanin synthesis in skin of fish and amphibia and the relief of vitiligo (27,44-47,197); (3) increase of basal metabolic rate (123); (4) effects on carbohydrate metabolism (123); (5) antidiuresis (123); and (6) effects on dark adaptation of the eye (123). With the exception of the last two all merit serious consideration. The antidiuretic activity once claimed for "B"-containing extracts is now generally agreed to have been due to contamination of the extracts with neural-lobe substance. The extensive claims related to the effect on dark adaptation of "B"-containing extract on the eye and the related claims that the pituitary and circulation of nocturnal birds contain more "B" than day-flying ones, are now merely interesting ideas, since recent work has shown the inadequacy of the methods used by the authors claiming these effects.

#### 1. Expansion of Chromatophores

This property is the best known attribute of "B"-containing extracts and is considered more fully in the preceding section. With the reservations noted in that section, injection of extract by any of the usual routes leads to sufficient concentration of "B" in the circulation to expand the melanophores, provided no strong direct peripheral nervous intervention is acting in contrary fashion (page 492). In this section it is appropriate that we consider the action of extracts prepared in different ways. Hypophysectomized amphibia or elasmobranchs are pale, with contracted melanophores. Simple aqueous extracts of dogfish intermediate lobe (*i.e.*, devoid of neural-lobe properties) injected into pale intact or hypophysectomized amphibians (or elasmobranchs) cause a rise of m.i. and this gradually subsides as the excitant is destroyed by the tissues (page 493). Since the effector speed of *Xenopus* melanophores has been accurately determined (154) we can roughly estimate from the time-m.i. graph the concentration of hormone in the circulation at various times. Fig. 19 shows that there is a quantitative relationship between the amount of pigment to be activated and the amount of excitant needed to cause melanophore expansion. After injection of an extract of similar "B" content, but containing pressor activity, the peak m.i. attained is not significantly different but pallor is more rapidly attained. Caustic soda treatment of unfractionated posterior-lobe

extracts modifies their melanophore-excitant properties in at least two ways: (a) the melanophore-expanding potency is increased, as judged by peak m.i. attained, (b) there is an increased duration of response when sub-

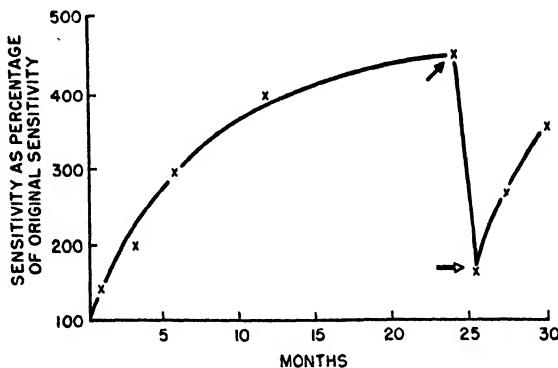


FIG. 19.—Graphic representation of the effect of "background" on the sensitivity of *Xenopus* to injection of "B"-containing extracts. All animals kept on a black "background" for several weeks before experiment. For the first 24 months of the experiment toads kept on a white "background." At → they were transferred to a black "background." At ⇒ they were transferred to a white "background." Points are the average sensitivity figures from same 12 toads. Sensitivity estimated at intervals shown by injecting each toad equilibrated on a white background with same dose of freshly made extract from the same sample of posterior-lobe powder kept in a desiccator at 0°C. (124)

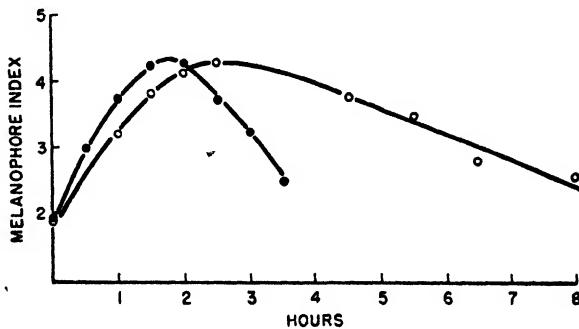


FIG. 20.—Completely hypophysectomised *Xenopus*. Responded to ox posterior-lobe extracts. All injections 1 ml. 15°C.—●—untreated aqueous extract. —○—same extract boiled with caustic soda. Half the above dose. (123)

maximal doses are injected (123) (Fig. 20). Work on isolated skin of *Scyllium* shows that both effects are exerted peripherally (225).

The increased *potency* has usually been attributed to destruction of pressor activity by the caustic soda treatment, but we have shown that the

degree of *potentiation* brought about by caustic treatment is not correlated with the pressor content of the extract before treatment, and that *increased duration of response* after caustic treatment may be obtained both from extracts with a high initial pressor content (ox) and extracts with little, if any, pressor properties (dogfish). We conclude that a substance(s) is present in posterior-lobe extracts which after treatment with alkali modifies the melanophore response evoked by "B" in at least two ways (122,123). We now have evidence which shows that increased duration of response is not an all-or-nothing phenomenon (124b), and which suggests that potentiation is a separate process from that causing increased duration of response (*vide infra*). We have no direct experimental evidence to show how the increased duration of response is brought about but it is probably due to a

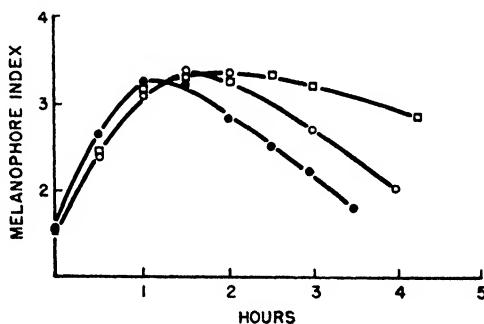


FIG. 21.—Completely hypophysectomized *Xenopus*. To show the duration of response evoked by three different B-containing extracts in doses that evoke the same rise of index. All injections 1 ml. 15°C. —●— Pitressin containing 0.004 International pressor Units. —○— carbon-extracted "B." —□— whole posterior lobe after treatment with caustic soda. (123)

reduced rate of destruction by the tissues. Hence to save periphrasis we meanwhile call it "protection." Comparison of the duration of effect evoked by various caustic-treated extracts shows that there may be different degrees of protection and which seem to be correlated with the amount of inert protein in the original (Fig. 21). Evidence that the potentiation is a separate phenomenon falls under three heads: (a) If carbon is added to a potentiated protected extract and elution is effected with glacial acetic acid, a "B"-containing extract is obtained that exhibits only very slight protection but is fully potentiated (123). (b) We have tried heating a crude extract to 99°C. for different lengths of time at different pH values in an effort to obtain evidence for or against the hypothesis that potentiation and protection are separate phenomena. Some loss of activity occurred if heating was continued for 10 minutes at pH 13.0 and over, and at pH 13.0 treatment for 3 minutes resulted in maximum potentiation and pro-

tection. No change occurred after a 3-minute treatment below pH 12.5. Between pH 12.5 and 13.0 potentiation and protection occurred together and no convincing evidence was obtained for their being separate phenomena. Evidence was obtained, however, when the effects of caustic treatment on crude extracts from different original material were compared. A method for deducing an index of protection was devised and the following results obtained (124b).

Animal	Extract	Potentiation	Protection
Ox . . . . .	Posterior lobe	2.0	1.8
Ox . . . . .	Anterior lobe	1.2	2.6
Dogfish . . . . .	Posterior lobe	3.2	1.8

(c) *Xenopus* equilibrated on a white background for a short time exhibits both potentiation and protection, but animals equilibrated under these conditions for 2 years sometimes exhibit only protection.

Posterior-lobe extracts cause expansion of erythrophores as well as melanophores, e.g., ventral reddening of *Phoxinus*. Some workers believe that two separate excitants are involved. Astwood (5) found that treatment with alkali potentiated the melanophore-expanding property of his extract but reduced its erythrophore activity to about one-tenth. We have tested (123) our own extracts, after treatment with caustic soda, on *Phoxinus* and could find no definite evidence that separate excitants are involved, since in our hands the *Phoxinus* test was found to be unreliable for quantitative work.

## 2. Increase of Melanin Synthesis

There is strong evidence for the participation of "B" in melanin formation. When chromatically active animals are maintained for long periods on a black background (maximum blood concentration of hormone) there is an absolute increase in the amount of melanin in the skin; on a white background there is an absolute loss (27,197).

More direct evidence comes from *in vitro* experiments. Certain aromatic groupings can be converted by oxidation and condensation into dark-brown or black pigments called melanins, varying in chemical composition but all characterized by a marked resistance to chemical change. The work of Furth, Bloch, Raper, and co-workers (8,54,166) has shown that tyrosine is the probable precursor of melanin in skin and that dihydroxyphenylalanine (dopa) is the first product formed from tyrosine in the process of melanin formation.

Fostvedt (42) has shown that the tyrosine-tyrosinase oxidation system in the production of melanin *in vitro* is accelerated by "B," but finds no such

acceleration of the autoxidation of dopa. Vilter (213) found local hyperpigmentation of the ear of rabbits after implantation of dry extracts of the hormone, and quoted Watrin, who found local hyperpigmentation in man after subcutaneous injection of posterior-lobe extract. More recently, Fournier (44-47) and co-workers, report that treatment of vitiligo with the hormone has proved successful in eight of eleven patients. Improvement of areas remote from the site of intradermal injections suggest a systemic action of the extract.

### *3. Increase of Basal Metabolic Rate*

Posterior-lobe extracts evoke a very definite metabolic effect in the absence of the thyroid. O'Donovan and Collip (154) observed an immediate metabolic stimulation in mammals as a result of injecting extracts rich in "B." The active principle could be distinguished from the thyrotrophic, adrenotrophic, and growth hormones of the anterior lobe and from neural-lobe activities. It was similar to "B" in its resistance to alkalis and in its anatomical distribution. However, more recent work has provided evidence which militates against the view that these effects are due to "B" itself. Teague (198) observed that preparations rich in "B" obtained from various sources and prepared by different methods vary considerably in their effect on oxygen consumption and, more important still, he found that one extract subjected to tryptic digestion produced even more consistent metabolic stimulation than the original extract. Our own purified extracts do not contain a metabolic stimulant when tested on mice in doses up to 50 I.U. "B." Teague's observation that metabolic stimulation is still a property of posterior lobe extracts after tryptic digestion shows that the substance responsible is not "B."

### *4. Effects on Carbohydrate Metabolism*

The following effects on blood sugar have been attributed to various posterior-lobe extracts:

*a. Hyperglycemia or Hypoglycemia in otherwise Untreated Animals.* The literature to date permits no definite conclusion as to what specific constituent of posterior-lobe extracts is responsible for the hyperglycemic or hypoglycemic actions of general posterior-lobe extracts (123). The available data imply that: (1) the effects are not attributable to the pressor, oxytocic, or melanophore-expanding activities as such; (2) they may be associated with any of the above three according to the method of their preparation; (3) the site of injection has important effects; (4) if the effects are due to specific substances, as distinct from a mixture of proteins, the fact that the same extract may be effective in one species and not in another means that, to be effective, the substance(s) must be injected in a definite combination with protein for each species.

*b. Antagonism to Hypoglycemia Evoked by Insulin Injections.* Experiments on the injection of insulin and posterior-lobe extracts indicate that the latter may antagonize the hypoglycemic action of the former in at least two ways. Burn (13) showed that, when whole posterior-lobe extract and insulin were injected subcutaneously and simultaneously, hypoglycemia did not occur. Gurd (81) obtained 40% abolition of insulin hypoglycemia in rabbits by simultaneous subcutaneous injection of 30 units Pitocin, and 80% abolition by 30 units Pitressin. If Pituitrin is injected some hours prior to insulin injection it has no inhibitory effect. Griffith (73), as a result of experiments employing posterior-lobe extract (Infundin) injected subcutaneously, and insulin injected subcutaneously or intravenously, has concluded that hypoglycemia does not occur after subcutaneous administration because peripheral vasoconstriction prevents the insulin from reaching the circulation in adequate amounts.

In contrast to this type of indirect action some workers have reported that posterior-lobe substance can antagonize the effect of insulin *when both are injected directly into circulation*, or when pituitary extract is injected intravenously after insulin has been permitted time to reach all tissues. Here again it is not possible to assign this inhibitory action to pressor, oxytocic, or melanophore-excitant activities as such. Thus Geiling, De Lawder, and Rosenfeld (62) noted "prompt and marked hyperglycemia" after intravenous injection of Pitocin (1 ml.) or Pitressin (0.5 ml.) into insulin-treated dogs. Ellsworth (37) found that Postlobin-O antagonized insulin hypoglycemia in the dog, but Postlobin-V was ineffective. Pituitary injections were given 5-30 minutes after intravenous injection of insulin. Griffith (73) concluded that "no satisfactory answer can yet be given to the question of the existence of a direct antagonism between insulin and posterior-lobe extract."

Evidence for the possible implication of "B" is derived from two sources. Young (227) was able to abolish the action of 2 units of insulin (crystalline) given intravenously or subcutaneously to pituitary-treated rabbits fasted 21 hours. Anterior-lobe extracts were given subcutaneously at the beginning and at the sixteenth hour of the fast. With his best extracts a dose equivalent to about 1-2 g. dried anterior-lobe powder was effective. He was able to show that the "glycotropic" action was not due to prolactin, thyrotrophin, or gonadotrophin. His extracts were probably contaminated with "B" and he did not eliminate this as the responsible agent. The fact that he found Pituitrin ineffective makes it improbable that "B" was the substance involved. Neufeld and Collip (149-151) reduced or prevented the hypoglycemic action of 6 units of insulin in fasted rabbits, by preliminary treatment with an anterior-lobe extract or (only one experiment recorded) alkali-treated posterior-lobe extract. The doses used represented

about 0.5–1.0 g. fresh gland tissue. Though they suggested that the anti-insulin effect might be due to "B," no assays for this excitant were disclosed. Working with a variety of extracts in different states of purification we concluded that any anti-insulin effects of posterior-lobe extract could not be attributed to "B." Our own purified extracts of "B" exert no anti-insulin effect (123).

c. *Antagonism to Hyperglycemic Action of Adrenaline.* Relatively little work appears to have been done on the adrenaline "antagonist." Burn (13) found that subcutaneous injection of posterior-lobe extract (Infundin) simultaneously with adrenaline diminished the hyperglycemic effect of the latter. Gurd (81) found that adrenaline hyperglycemia in the rabbit was diminished by doses of 30 units of vasopressin or oxytocin, the former being more effective. Neufeld and Collip (172) claimed that their insulin-antagonizing extracts also antagonized adrenaline in fasted rabbits and concluded that "a more pronounced antagonism to insulin hypoglycemia and adrenaline hyperglycemia resulted after prolonged treatment; the adrenaline antagonism appeared to be more defined than that to insulin." In a later paper they reported the effect of posterior-lobe extracts in fed rabbits given subcutaneously 45 minutes and 0 minutes before subcutaneous injection of 0.1 mg. adrenaline. All the extracts—alcohol, 0.25% acetic acid, and water extracts—were boiled 10 minutes with N/10 sodium hydroxide to destroy pressor and oxytocic action, but apparently no "B" assays were made. Their doses were equivalent to about 1 g. original tissue. Fairly consistent antagonism to adrenaline was observed, even when adrenaline was given intravenously and the extract given subcutaneously or intravenously. Commercial oxytocin was ineffective. Our own experiments (123) show that "B"-containing posterior-lobe extracts inhibit the hyperglycemic effects of adrenaline and more significant still, that our most highly purified extract, which contains no trace of any other activity, inhibits adrenaline hyperglycemia. It is still too early to make a definite claim that "B" itself inhibits adrenaline action, but the fact that the hyperglycemia evoked by 0.1 mg. adrenaline is largely inhibited by subcutaneous injection of 0.25 mg. (Fig. 22) or 0.1 mg. of a more recent product has encouraged us to examine whether this phenomenon is of physiological significance.

#### D. BIOLOGICAL ASSAY

When a "B"-containing extract is injected into pale test animals the melanophores expand and the skin darkens. The skin then gradually gets paler as the melanophores contract. Potency may be measured by the following criteria: (1) minimal amount necessary to effect a change, (2) degree of darkening, or (3) the time the test animal remains dark. The

first is time-consuming and inaccurate; and from what has been said on page 496, it is evident that the third measures something else as well, so only the second criterion is acceptable. Actual measurement of skin darkening or melanophore expansion can be done by naked eye estimation of the color of the skin or by microscopic observation of the melanophores. The objections to the former, as well as to Hill's photoelectric method of recording it, have been fully stated elsewhere (124,148). The overriding

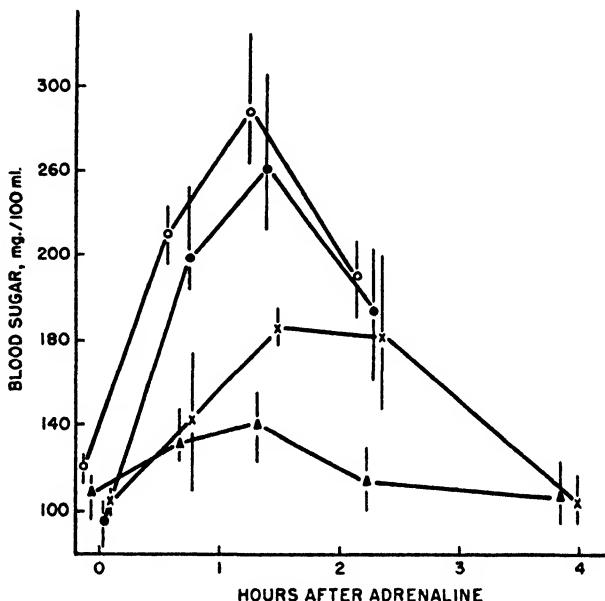


FIG. 22.—Effect of L.R.W.<sub>1</sub> on adrenaline hyperglycemia. Three male rabbits fasted 21 hours before subcutaneous injection of 0.1 mg. adrenaline. "B" injected subcutaneously in two equal portions, 45 and 0 minutes before adrenaline. Vertical lines indicate maximum and minimum values observed. ●, ○ Controls (adrenaline alone). × 10,000 units "B" and ▲ 20,000 units "B." Injections were made in the order —●—, —×—, —○—, —▲—. (122)

objection is the variation in melanophore density among animals, which may be so great that an animal with few but fully expanded melanophores is paler than an animal with abundant contracted ones. Hence quantitative results of real value can only be obtained by direct estimation of the degree of expansion of the melanophores themselves (Fig. 23).

Any animal which changes color in response to "B"-containing extracts could be selected as a potential test animal. It is not surprising therefore that several methods have been proposed (124). Chief of these we may note: (1) injection into the dorsal lymph sac of *Rana* or *Xenopus*, (2) in-

jection of extract into *Anolis*, (3) immersion of isolated frog's skin in the extract to be assayed, (4) perfusion of isolated frog's limbs and (5) injection into *Phoxinus*. The first of these is best and will be considered later. The shortcomings of the others can be summarized as follows: The method employing *Anolis* depends upon macroscopic observation of the skin, the disadvantages of which have been outlined above. Immersion of isolated skin in extract can only give, *at best*, a very rough figure; as usually employed it is a qualitative test only and even then is open to serious error in the presence of extraneous matter. Using dogfish skin we have found it possible to discriminate between doses over a very small range but we do not recommend the method for assay. Perfusion of isolated limbs is again,

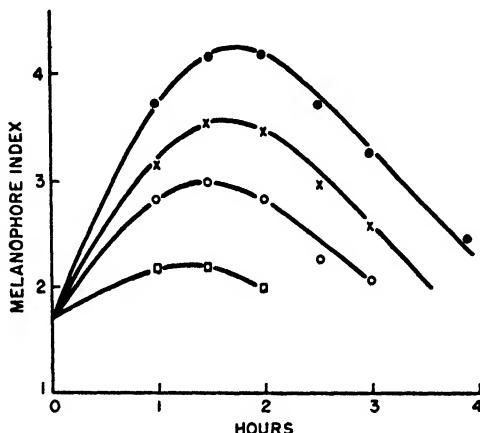


FIG. 23.—Response of intact animals to graded doses of a "B"-containing extract (supplied by Prof. Stehle). 15°C., white background, 0.25 ml. injections into dorsal lymph sac. ● = 0.2 µg., X = 0.1 µg., ○ = 0.5 µg., □ = 0.025 µg. Stehle's powder.(124)

in practice, only a qualitative test because *each preparation can be used for only one dose level*; in our hands it does not discriminate well between doses. Hence it is at best limited to the minimal effective dose principle (*vide supra*). The test on *Phoxinus* depends on the expansion of erythrophores. Aside from the fact that it is still an open question whether crythrophores and melanophores are activated by the same pituitary autacoid, we have found the *Phoxinus* test quite unreliable (122). We conclude then that the only quantitative assay of value is based on injection into normal or hypophysectomized frogs (199) or *Xenopus*. The latter has material advantages which have been fully detailed elsewhere (124).

Accurate assay of "B" activity by our method is lengthy when compared to pressor or oxytocic assays. With these the effector speed is high so that many doses of standard and unknown can be injected and equated on the

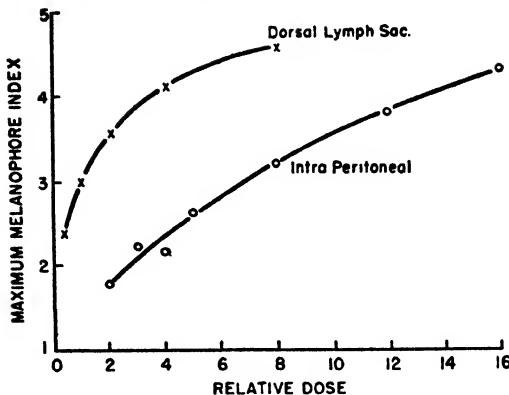


FIG. 24.—Dose-response curve of *Xenopus* (eighteen animals).  $\times$  Dorsal lymph sac;  $\circ$  intraperitoneal. (124)

TABLE XI (124)

RELATIVE "B" CONTENT OF FIRST AND SECOND INTERNATIONAL-STANDARD POWDERS<sup>a</sup>

Date	First International-Standard powder				Second International-Standard powder				Second International Standard as percentage of first International Standard	
	Group of 12 toads	Dose in milliunits	Max. m.i.	Average max. m.i. from 24 toads	"Relative dose" read from fig. 24	Group of 12 toads	Dose in milliunits	Max. m.i.	Average max. m.i. from 24 toads	
5/18/43	A	5.0	4.1	4.05	3.5	B	5.0	4.0	4.0	$\frac{3.2}{3.5} \times 100 = 91$
5/19/43	B	5.0	4.0			A	5.0	4.0	3.2	
5/20/43	A	2.5	3.2	3.2	1.25	B	2.5	3.2	3.15	$\frac{1.2}{1.25} \times 100 = 96$
5/21/43	B	2.5	3.2			A	2.5	3.1	1.2	
5/22/43	A	1.25	2.4	2.4	0.5	B	1.25	2.6	2.45	0.53
5/23/43	B	1.25	2.4			A	1.25	2.3	0.53	$\frac{0.53}{0.5} \times 100 = 106$

<sup>a</sup> Two groups of twelve intact *Xenopus* of approx. equal sensitivity, from same group as used for Fig. 24. Initial extractions of powder with dilute acetic acid as *British Pharmacopoeia*. Dilutions made with *Xenopus* saline. All injections 0.25 ml., dorsal lymph sac. All assays on white background with constant overhead light; 15°C.

same preparation in a short time. Melanophore speed is slow so that in a working day only one injection per toad can be given. By using four

groups, each containing six selected toads, and two appropriate doses (see Fig. 23) of both standard and unknown, a result accurate to 20% can be obtained in 1 day by reference to a dose-response curve (Fig. 24). To get 10% accuracy it is necessary to take 2 days for an assay, changing the groups of toads from standard to unknown and *vice versa* on successive days. In this way all toads are used for both unknown and standard. The two groups of toads are selected on two criteria: (a) similarity of response to a given dose, and (b) small number of melanophores<sup>4</sup> for easy

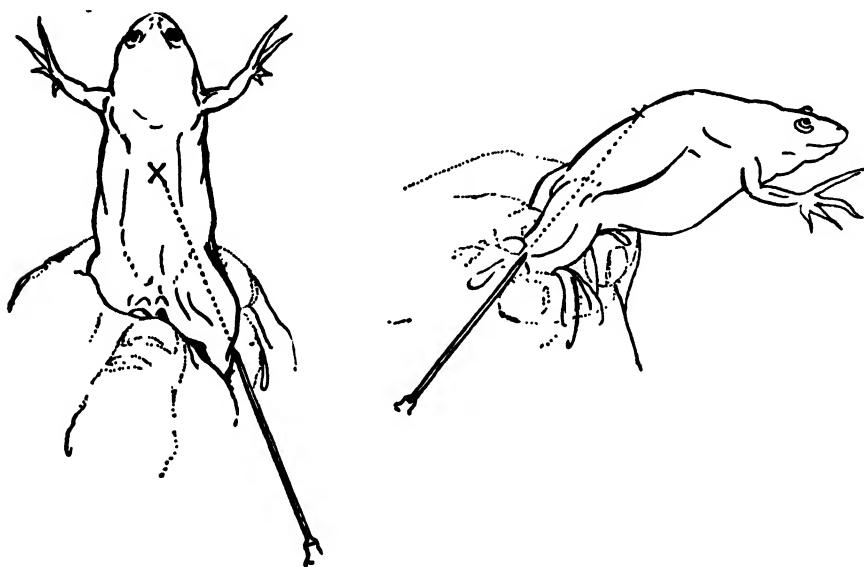


FIG. 25.—Method of injecting material into dorsal lymph sac of *Xenopus*. (124c).

reading. On the first day of the test, the standard is injected into the first group and the unknown into the second group, both at three dose levels. On the next day the procedure is reversed. Table XI shows the results of a typical assay. When assaying body fluids etc., hypophysectomized animals must be used for the final test because some fluids have the power to evoke endogenous pituitary secretion. For routine assays of pituitary extract intact animals have the advantages of being easier to maintain and less susceptible to extraneous toxic matter.

Since many people experience difficulty in making a successful dorsal lymph sac injection there is included a drawing (Fig. 25) of correct procedure.

<sup>4</sup> This can be adjusted (p. 498).

### E. PURIFICATION AND CHEMISTRY

Purification here implies removal of inert protein and separation of the excitant from other pharmacologically active substances in the gland. With regard to the latter the ideal procedure would be to use a gland from, e.g., the elasmobranch, in which there are no significant amounts of neural tissue, or an animal such as a bird, in which "B" is located in the anterior pituitary (118). With the latter, most contaminating anterior-pituitary hormones could be destroyed by heat treatment. In practice limitation of supply makes either one impossible, so we use whole posterior-lobe material from domestic animals and depend on separating "B" from pressor and oxytocic properties by chemical or physical methods. Broadly speaking three methods have been used:

#### 1. Alkali Treatment of Whole Posterior-Lobe Extract

Simple aqueous or dilute-acid extracts of ox posterior lobe contain neural-lobe properties as well as "B." The former can be destroyed by boiling an extract at pH 13.0 for 10 minutes. This method is probably sufficient for most routine purposes but the final extract contains considerable inert matter and the "B" properties will have been altered in the two ways discussed on page 496. A modification of this method, due to Zondek and Krohn (228), gives a purer product (in the sense that inert protein is removed) but presumably the final extract will contain "B" in modified form. The initial dilute-acid extract of dried powder is evaporated to dryness and the residue extracted three times with absolute alcohol. The alcohol solution is evaporated to dryness and the solid dissolved in water. The solution is then treated with caustic soda to destroy pressor and oxytocic properties. The authors state that further purification can be effected by adding ether, acetone, or ethyl acetate to a concentrated alcoholic solution of the hormone; impurities remain in solution while the activity is precipitated. Owing to their method of standardization it is impossible to assess the purity of their product as compared to those set out below. They state that 0.001 mg. of their most active preparation will definitely cause ventral reddening in *Phoxinus*. Similar amounts of both Stehle's and the authors' extracts will also evoke this effect but no comparative assays have been made (122).

#### 2. Fractional Precipitation

The original "vasopressin" produced by Kamm *et al.* (115) contained large amounts of "B," while their "oxytocin" had little. Subsequently commercial samples of the Parke-Davis pressor fraction exhibited less "B" activity, but how the "B" was removed has not been disclosed. Neither Stehle's pressor nor oxytocic fractions (page 462) have appreciable quanti-

ties of "B," and Stehle (190) has described how it can be recovered from the original mother liquor. When a dilute acetic acid extract of commercial posterior-lobe powder is concentrated and then treated with 5–10 volumes of alcohol, almost all the pressor and oxytocic hormones are carried down in the precipitate but melanophore hormone remains in solution (*cf.* Table V). The alcoholic solution is evaporated to dryness; "B" is dissolved from it with methanol and is reprecipitated with ethyl acetate. This precipitate is dried and re-extracted with methanol; the methanol-insoluble material is dried *in vacuo*. It is 25 times standard as regards "B" activity (assayed on the frog), and contains less than 0.5 unit pressor and oxytocic activity/100 units "B."

This procedure of Stehle's is particularly interesting in emphasizing the fact that at different stages of purification a single substance, in this case methanol, can be used as a precipitant and a solvent for the activity.

### 3. Fractional Adsorption and Elution

We have described a number of procedures for using carbon adsorption to obtain "B"-containing extracts (122). These have been chiefly designed to throw more light on the components involved in "potentiation" and "protection," but they have yielded the purest "B"-containing extract yet described.

*L.R.W.<sub>1</sub>.* A dilute-acid extract<sup>5</sup> of the original powder is treated by heating with alkali. The activity is adsorbed on charcoal, eluted with glacial acetic acid, and precipitated with ether. The final powder usually contains about 150 I.U./mg. and is "protected." It is practically free from pressor and oxytocic activities, containing less than 1 unit/mg. of either. Yields up to 60% have been obtained by this method, and potencies as high as 200 I.U./mg. have been attained with good original material.

The final powder is stable for many years if stored dry in a sealed glass ampule. Solutions are stable for some months at room temperature if stored in evacuated ampules, but keep for only a few days in open tubes.

*L.R.W.<sub>2</sub>.* This extract is made in a similar way to L.R.W.<sub>1</sub> except that alkaline treatment is omitted. The final powder is not, however, as potent (25 I.U./mg.) and is contaminated to a greater extent with pressor and oxytocic activities (2–5 I.U./mg.). Its stability is similar to L.R.W.<sub>1</sub>. The extract is unprotected and alkaline treatment of it results in neither "protection" nor "potentiation."

One of the greatest difficulties in preparing purified extracts of "B" is the acquisition of sufficient original material of reasonable potency. Com-

<sup>5</sup> If a potent powder approximating I.S.P. is used, the isoelectric point of the activity in a simple extract is about pH 4.1 and much more inert matter can be removed by a second heating at pH 4.7. A good procedure is to extract at pH 3 to 3.5, filter off inert insoluble material, adjust to pH 4.7, reheat to 70°C., and filter.

mercial posterior-lobe powders are made from ox tissue and often contain as little as 10% "B," while containing 60% pressor and oxytocin when compared to the International Standard, *i.e.*, only 0.2 I.U."/mg. We have found that horse and pig material is much more potent than ox, a horse posterior-lobe powder having been made by us containing four times as much "B" as the International-Standard powder. Unfortunately it is difficult to get horse and pig material in sufficient quantity for general use. The extracts described have been made from commercial ox posterior-lobe powder containing about 0.8 I.U./mg. Better material than this yields better products.

Little is known about the chemical nature of the substance responsible for the melanophore-expanding property of pituitary extracts. It is fairly heat stable, soluble in water, destroyed by trypic but not by peptic digestion, and easily extracted from glandular material with acid or alkali. Similar yields are obtained if extracts are made at standard strength by the standard method at any pH between 3.0 and 12.0, though pH values higher than this modify the activity (124b). The isoelectric point in a standard extract of standard powder is about pH 4.1 (225). Dreyer and Clark (32) found that a collodion ultrafiltrate was about 25% as strong as their original extract in oxytocic activity but less than 5% as strong in melanophore activity. On the other hand, Zondek and Krohn (228) found that their extract dialyzed more rapidly than an oxytocic preparation. The different results may be due to inadequate assay techniques or to adsorption of the active principle to different carriers (page 468). The molecular weight of the active substance in most extracts is probably not very much greater than 2000. The activity is soluble to some extent in methyl, ethyl, propyl, and butyl alcohols and insoluble in ether, acetone, and ethyl acetate (191). It is easily adsorbed from solution by carbon and kaolin.

Stehle (192) derived three unlike fractions from a posterior-lobe extract which were indistinguishable biologically or chemically. These fractions were all "picrates" and were 73 times as potent as standard powder when calculated to a picric acid-free basis. Tyrosine was present to about 4.7% and arginine and cystine were also present. Tryptophan was measured by the method of May and Rose (135) using casein as a standard and was present to the extent of 5% if it is assumed that casein contains 1.5% tryptophan. Since this amino acid is not a constituent of pressor and oxytocic fractions, Stehle (193) suggests that the tryptophan content be used as a basis for comparing melanophore-expanding preparations.

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## CHAPTER IX

### Chemical Control of Nervous Activity

#### A. Acetylcholine

By DAVID NACHMANSOHN

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## I. Introduction

For any attempt at analyzing the mechanism of nervous function, it is essential to keep in mind two characteristic features: the high speed and the small amount of energy involved. Nerve impulses may be propagated at a speed up to 100 meters per second. Electrical signs of nerve activity seem to indicate that, during the passage of the impulse, the events at any one point of the neuronal surface membrane may occur within a fraction of a millisecond, probably close to 100 microseconds. The initial heat per gram and impulse is of the order of magnitude of 1 erg, or less than one-tenth of a millionth of a small calorie. It is not surprising that, for more than a century, only the electrical signs of this event were studied, since for these manifestations sufficiently sensitive methods were available. However, even for the electrical studies, a really adequate instrument became available only by the introduction of the cathode ray oscilloscope. The development by A. V. Hill of methods of such a high degree of perfection that heat production could be demonstrated and even the different phases analyzed is one of the most remarkable achievements of modern physiology.

Although physical methods only were available for studying nerve activity, the necessity of an understanding of the underlying chemical mechanism was early recognized. The small electric currents which propagate the nerve impulse cannot derive the energy from the stimulus itself as in the case of the sound wave. This energy must be supplied locally by a "propagated disturbance" as Lucas and Adrian pointed out, *i.e.*, by a local chemical change (119). Hill's Liversidge lecture, "Chemical Wave Transmission in Nerve," is a most lucid and stimulating formulation of the whole question and a challenge to biochemists to attack the difficult but interesting problem (92).

While the peculiar features of nerve function create many difficulties even for recording with physical methods, a chemical analysis of substances which presumably appear and disappear within microseconds and in infinitely small amounts offers even more serious difficulties. How-

ever, instead of measuring the chemical substances involved, another way of approach of analyzing a cellular function is possible, *viz.*, a study of the enzymes which catalyze the reactions of chemical substances in this process. Many enzymes are relatively stable and may be extracted from the cell. In this way, it may be possible to reconstruct events occurring in the living cell *in vitro*. It is true that enzymatic reactions measured *in vitro* indicate only potential rates. But if the reactions catalyzed by the enzymes studied are correlated with events in the living cell recorded by physical methods, such an analysis may be as conclusive as direct measurements of the reacting substances. Stimulated by the pioneer work of Otto Meyerhof and A. V. Hill, valuable information has been obtained in this way about the mechanism of muscular contraction, by the combined efforts of many investigators. A similar approach appeared to be the most promising way of studying the chemical reactions underlying conduction.

Two compounds have been specifically connected with nerve activity adrenaline and acetylcholine. In 1904, Elliot suggested that adrenaline may be released from the sympathetic nerve endings and act as a transmitter of the nerve impulse to the effector organ (71). He based his theory on the similarity of the effect of adrenaline to the stimulation of sympathetic nerves. Since that time, the idea of neurohumoral or chemical transmission of nerve impulses increasingly attracted the interest of physiologists. Dixon assumed a similar mechanism of transmission for parasympathetic nerves acting on the effector organ by the release of a muscarinelike compound (65). Howell and Duke observed the liberation of potassium following stimulation of the heart and thought that the vagus may affect the heart in this way (96).

The knowledge of a relation between acetylcholine and parasympathetic nerves was initiated by Hunt and Dale. Hunt and Taveau in 1906 described extremely powerful effects of acetylcholine. They observed that the ester decreases the frequency of the heart in a manner similar to the effect of vagus stimulation and already discussed the possibility of the occurrence of the ester in the body (98). Later, in 1918, Hunt found that tissues are much more sensitive to acetylcholine after treatment with eserine (97). In 1914, Dale considerably extended the observations of Hunt and Taveau on pharmacological effects of acetylcholine and other choline esters (60). He divided the actions of acetylcholine into two groups: (1) the muscarinelike action reproducing essentially the effects of parasympathetic stimulation, and (2) a nicotine-like action which appears after elimination of the muscarinelike action by atropine; the nicotinelike action was similar to the effect of stimulation and subsequent paralysis of sympathetic ganglia and also affected voluntary muscles.

Although both Hunt and Dale had envisaged the possibility of a physiological role of acetylcholine, there was no evidence for this assumption. But in 1912, Weiland, in Magnus' laboratory, showed that a substance is released from the intestine which acts upon another intestine like nerve stimulation (201). This substance was identified by LeHeux, in 1919, in the same laboratory, to be at least 75% choline (105a). Among the choline esters tested, LeHeux found acetylcholine to be the most efficient (105b). Further progress was made when Loewi, in 1921, found that, following vagus stimulation, a substance appeared in the perfusion fluid of the frog heart which when applied to a second frog heart had the same effect as vagal stimulation (115). He called this substance "*Vagusstoff*." By a series of observations in different laboratories, this *Vagusstoff* was later identified with acetylcholine, as might have been assumed on the basis of the work of Magnus and associates and the observations of Dale concerning the powerful action of this ester on cells innervated by the vagus. Dale and Dudley isolated acetylcholine from the spleen of ox and horse and thus offered evidence that acetylcholine actually occurs in the body (62). From all these findings of the different schools, the picture emerged that acetylcholine is the chemical mediator of the nerve impulse at parasympathetic endings and that adrenaline or an adrenalinelike substance has the same function at sympathetic endings, as proposed first by Elliot and supported mainly by the work of Cannon and associates (39).

In 1933, Dale tried to extend the idea of chemical mediation of nerve impulses to the ganglionic synapse and to the neuromuscular junction. His evidence was built essentially on experiments of the same type as used in the case of the peripheral autonomic system. The two fundamental facts were: (1) the excitability of the effector cell by small amounts of acetylcholine, and (2) the appearance of acetylcholine in the perfusion fluid of the synaptic junctions following the stimulation of the presynaptic nerve fibers (preganglionic and motor nerves, respectively) (32, 61).

But, whereas the theory of chemical mediation of nerve impulses appeared acceptable to many physiologists in the case of autonomic nerves acting on their effector organ, this concept, when applied to synapses and neuromuscular junctions, was less satisfactory and encountered increasing opposition. Besides a great number of difficulties and contradictions, which were partly reviewed by Eccles (67) and which have increased continuously since then, there were two main objections. The first was the time factor. This factor was of lesser importance in the case of the slowly reacting cells innervated by the autonomic nervous system. But the transmission of nerve impulses across the neuromuscular junctions and synapses occurs within milliseconds. No evidence was avail-

able that the chemical process can occur at the high speed required and Dale admitted this difficulty. The second objection was still more fundamental. The work of Sherrington and his school indicates that the excitable properties of the central neurons are similar to the excitable properties of the peripheral nerves, *i.e.*, the axons (80). The problem has been scrutinized by Erlanger (74) in the Symposium on the Synapse, in 1939. Analyzing some of the peculiarities attributed to the synapses, *viz.*, latency, one-way transmission, repetition, temporal summation or facilitation, and transmission of the action potential across a nonconducting gap, he points out that all these phenomena can also be demonstrated on fibers. Gasser (82) arrives at a similar conclusion. The facts based on the electrical signs of nerve activity thus make it unnecessary to assume, especially in view of similar time relations, that any condition exists at the synapse which differs in any basic way, except quantitatively, from that found in the peripheral axon. The idea of a chemical mediator, released at the nerve ending, diffusing through the nonconducting medium, and acting directly on the second neuron or muscle, thus appeared to be unsatisfactory in many respects. In this impasse, a new approach appeared necessary. For reasons outlined above, the study of the enzymes connected with acetylcholine metabolism seem to offer hope of obtaining more satisfactory information.

Investigations on these lines have shown that the hypothesis of chemical transmission as proposed originally must be modified. A great variety of facts have accumulated suggesting that the release and removal of acetylcholine are intracellular processes occurring in the neuronal surface membrane and are a necessary link in the chain of reactions which generate the small electric currents conducting the impulse (80,81,136, 146-149, 167a).

## II. Acetylcholine Esterase

Acetylcholine esterase (ACh esterase) is the enzyme which hydrolyzes acetylcholine into choline and acetate and thereby inactivates the ester. This name has been recently proposed by Augustinsson and Nachmansohn (15). From the studies of this enzyme emerged six essential features of physiological significance.

### A. TIME FACTOR

The most important prerequisite for any assumption connecting a chemical reaction directly with electrical manifestations is the extremely high speed of such a reaction. On the basis of the electrical signs, it must be postulated that any chemical event underlying the electrical should occur within less than 100 microseconds. The most outstanding feature of the ACh esterase is the fact that the speed at which it hydrolyzes acet-

ylcholine satisfies this postulate. The "turnover number" has been estimated with an enzyme solution prepared from the electric tissue in the following way: The enzyme extracted from the electric organ of *Electrophorus electricus* has been purified by fractional ammonium sulfate precipitation to a degree where 1 mg. protein is able to split about 20 g. acetylcholine/hour. By high-speed centrifugation, a further separation of the proteins has been achieved. The enzyme solution obtained finally in this way was able to hydrolyze about 75 g. acetylcholine/mg. protein/hour (188). The analytical run in the ultracentrifuge showed that only one component was present; the molecular weight was estimated on the basis of the sedimentation rate to be close to three million. On the basis of these data, the turnover number is of the order of magnitude of twenty million/minute. Such a figure indicates that one molecule of enzyme may split one molecule of acetylcholine in about 3 to 4 microseconds. Even if by a more accurate determination of the molecular weight some corrections may become necessary, this speed is sufficiently high for the assumption that this reaction may determine the electrical manifestations.

Any mechanism of a living cell is, on the basis of all available evidence, connected with a whole chain of reactions. Several compounds have been associated with nerve activity and the extra oxygen uptake observed is sufficient evidence that the passage of the impulse must be associated with a great number of reactions. But for no other chemical reaction known to be associated with nerve activity has a comparable speed been demonstrated, and, for this reason, it is the only reaction which at present can be associated directly with the electrical events.

### B. CONCENTRATION AND DISTRIBUTION

In addition to the high rate of its hydrolytic action, the enzyme must be present in concentrations adequate to account for significant amounts of acetylcholine split per unit time and unit tissue. Whatever the speed of the reaction, a very low concentration of the enzyme in nerve tissue would be prohibitive for the assumption of its playing an essential role. Extensive studies on the concentration and distribution of ACh esterase in conductive tissues have shown that significant amounts of acetylcholine may be split per gram tissue within a millisecond, *i.e.*, within a period of time during which the impulse passes (137,138,142). The concentration of the enzyme is high in all nerve tissues. Nerve fibers are capable of hydrolyzing amounts of acetylcholine ranging usually from 5 to 50 mg./g. fresh tissue/hour. In a few cases the concentration is below or above these figures. In regions where cells and synapses are located, the concentration rises to values several times as high as those found in

the nerve trunk. In the preganglionic fibers of the superior cervical sympathetic of cats, *e.g.*, the amount of acetylcholine which may be split per gram per hour is 50 to 70 mg. and rises to 400 to 600 mg. in the ganglion. In the abdominal chain of lobster, the values vary from 80 to 140 mg. in the trunk and from 180 to 300 mg. in the region where cells and synapses are present. In the gray matter of brain, the figures range in most cases between 30 and 400 mg., although here again they are in a few cases higher or lower. In the white matter, the values are usually below 10. Great variations are found in the different centers of the brain. The values vary also considerably from one species to another. In small animals (rats, rabbits), the values are markedly higher than in ox brain, whereas they are lower in the human brain. Table I shows the concentrations of ACh esterase in the nerve tissue of a variety of groups of animals and different species. Table II illustrates the variations of the concentrations in different brain centers. Only a few figures are included, but more are available and the number of observations is continuously increasing. Additional information may be found in Prosser's comprehensive review on the physiology of nervous systems of invertebrate animals (179).

In contrast to the variations between the different centers and the different species, the values for the same center and the same species are surprisingly constant. Fibers with a very thin myelin sheath, until recently considered unmyelinated, have generally a higher concentration than the so-called myelinated. In the ventral roots, the concentration is about twice as high as in the dorsal roots. To what factors all these variations are to be attributed is at present unknown.

### C. LOCALIZATION

The real significance of these concentrations can be properly evaluated only in connection with experiments on the peculiar localization of this enzyme in conductive tissues. The enzyme is not evenly distributed. This was first demonstrated in experiments on the frog sartorius muscle (122-124). A small fraction of this muscle near the pelvic end is free of nerve endings. By determining the concentration of ACh esterase in this part of the muscle, in the part containing nerve endings and in the nerve fibers, it was possible to demonstrate the presence of a very high concentration of the enzyme at the motor end plate. The number of end-plates in the sartorius of the species of frogs used was determined by Pézard and May (178). Therefore, the amount of acetylcholine which may be split during 1 millisecond at a single end plate could be calculated. This amount is about  $1.6 \times 10^9$  molecules of the ester. A similarly uneven distribution in mammalian muscle was demonstrated in experiments

**TABLE I**  
**CONCENTRATION OF ACh ESTERASE IN NERVE TISSUE OF**  
**VARIOUS ANIMALS**

**A. Brain**

Animal	ACh hydrolyzed, mg./g.tissue/hr.
<b>Mammals</b>	
Rabbit.....	80-100
Rat.....	80-100
<b>Birds</b>	
Fowl.....	250
Humming bird.....	300
Sparrow.....	300
Pigeon.....	240
<b>Amphibia</b>	
Frog.....	60-70
Turtle.....	80-100
<b>Fish</b>	
Goldfish.....	100-150
<b>Invertebrates</b>	
Squid (head ganglion)...	3000-4000

**B. Peripheral Fibers**

Mammals	
Dog	
Ventral roots .....	25-30
Dorsal roots .....	12-15
Sciatic .....	8-10
Optic .....	10-15
Sympathetic preganglionic .....	40-60
Sympathetic ganglia .....	150-200
Spinal ganglion .....	30-50
Cat	
Superior cervical sympathetic .....	60-70
Superior cervical ganglion .....	400-600
Postganglion (adrenergic) .....	25
<b>Amphibia</b>	
Frog	
Splanchnic (adrenergic).....	10
Sciatic.....	-10
<b>Invertebrates</b>	
Ray	
Optic nerve.....	2-4
Squid	
Fin nerve.....	4-7
Stellar nerve.....	2-4

on the gastrocnemius of the guinea pig by Couteaux and Nachmansohn (50,53). In the interior section of this muscle, the nerve spreads through nearly the whole muscle at one level only; it is situated in the midst of the muscle except for its entrance and its termination. In the middle third of this muscle, all nerve fibers as well as the endings are situated in the middle zone only. If this third is cut in slices with the freezing microtome, the slices near the upper and lower surfaces are virtually free of nerve endings; those of the middle zone contain a large number. As may be seen in Fig. 1, determinations of ACh esterase in these slices show a high concentration in the middle zone as compared to the low concentration in the nerveless zones. The results agree with the previous observations on the frog sartorius.

TABLE II  
VARIATIONS OF ACh ESTERASE CONCENTRATION IN DIFFERENT  
BRAIN CENTERS

Brain center	ACh hydrolyzed, mg./g. tissue/hr.			
	Rabbit	Dog	Ox	Man
Cortex . . . . .	60-80	20-50	20-30	12
White matter (gr. hemisphere)	.	3	2-3	.
Nucleus caudatus . . .	350	500-600	400	300
Nucleus lentiformis (putamen)	.	.	680	460
Cerebellum . . . . .	90-100	120-150	20-40	80
Optic thalamus . . . .	120	60	50	30
Pons . . . . .	130	70-80	...	60
Corpus quadrigemina, ant... .	250	140	100-120	60
Corpus quadrigemina, post... .	130	50	40	30
Retina . . . . .	.	150	140-200	.

The significance of these results becomes still clearer in connection with other observations. It was already mentioned that preganglionic fibers of the superior cervical sympathetic of the cat may split 50 to 70 mg. acetylcholine/hour, whereas the ganglion splits 400 to 600 mg. acetylcholine in the same period of time. Following the section of the preganglionic fibers, the concentration of the enzyme falls rapidly and is about 40% of the initial value at the time when the nerve endings have disappeared. Then the enzyme concentration remains constant for many weeks. Consequently, the amount of enzyme which disappeared must be attributed to the enzyme localized inside the fibers which disappeared during degeneration inside the ganglion. On the basis of these data, the concentration in the preganglionic fibers per unit tissue appears to be much higher inside the ganglion than that found in the same fibers before they enter the ganglion. The question arose how such an increase may

be explained. Since the preganglionic fibers have an enormous end arborization in the superior cervical ganglion, it appeared possible to assume that the strong increase of enzyme concentration may be related to the enormous increase in surface. It appeared then possible that the enzyme might be localized in the neuronal surface (50,53).

Evidence for this assumption was offered in experiments with the giant axon of squid. This axon became familiar to biologists after the brilliant work of Young (202), Schmitt, and their associates (22). From a squid

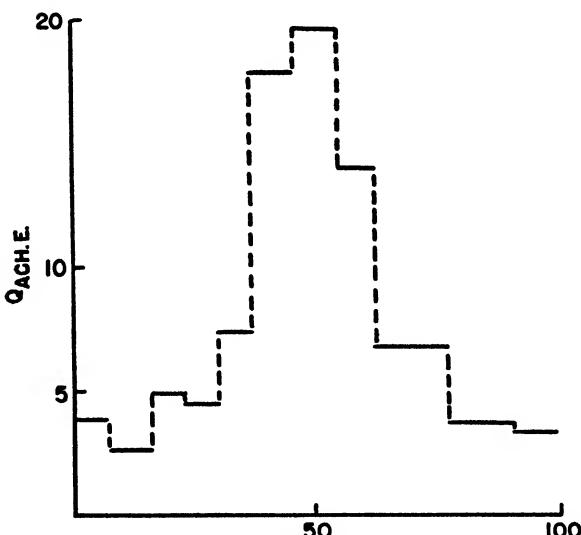


FIG. 1. Concentration of ACh esterase in the middle portion of the interior section of a guinea pig's gastrocnemius cut in eleven slices of similar thickness and weight. Each horizontal line corresponds to one slice and indicates its weight in per cent of total weight. Abscissas: Region from which tissue was obtained in terms of order of consecutive slices. Point 50 corresponds to center region where nerve endings are situated.

of average size, one readily obtains axons 0.4 to 0.8 mm. in diameter and 50 to 60 mm. long. Fig. 2 shows a cross section of this preparation. It is possible to extract the axoplasm from the axon and to study its compounds and enzymes separately from those in the envelope. The envelope is mostly connective tissue, but attached to it there are two membranes, each a few microns thick. The ACh esterase is localized nearly exclusively in the sheath. No ACh esterase activity was found in the axoplasm (26).

Bioelectrical phenomena are, as has been for a long time generally assumed, surface phenomena. Experimental evidence for this view was

recently offered by Hodgkin and Huxley (93,94) and Curtis and Cole (58) with observations on the giant axon of squid. By inserting electrodes into the interior of the axon, these investigators were able to measure directly the potential across the membrane and to follow its variations during activity. The potentials measured were even higher than previous estimations had anticipated. The exclusive localization of ACh esterase



FIG. 2. Portion of transsected stellar nerve of squid containing the giant axon (202). l. ax., large axon; s. ax., small axon; n., nucleus of sheath.

in the axonal surface is therefore interesting, especially in connection with the high concentration of the enzyme and the speed of the enzymatic reaction. It becomes even more pertinent in the light of the experiments on inhibitors of enzymes which split choline ester, to be described later. But it also gives a different meaning to the concentrations of the enzyme found in nerve tissue, since it is obvious that the concentrations measured per unit total weight do not indicate the actual concentration of the en-

zyme, which is limited to an extremely small fraction of the tissue. Consequently, the concentration must be very high there. The amount of acetylcholine which may be split per gram brain in 1 millisecond on the basis of the figures given above is of the order of magnitude of  $10^{11}$  to  $10^{15}$  molecules. It is of interest to estimate the surface area which could be covered by such an amount of acetylcholine metabolized. On the assumption that one molecule of acetylcholine may cover  $30 \text{ \AA}^2$ , 30 to 300 million  $\mu^2$  nerve surface may be covered by the amount of acetylcholine which may be metabolized in 1 g. brain in 1 millisecond.

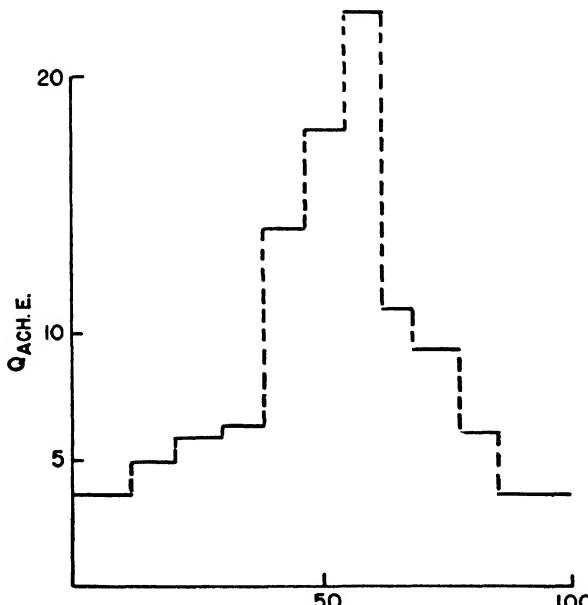


FIG. 3. Concentration of ACh esterase in the middle portion of the interior section of guinea pig gastrocnemius cut in eleven slices of similar thickness and weight, 14 days after section of the sciatic. Abscissus as in Fig. 1.

Another series of observations led to a better understanding and a more detailed analysis of the data obtained for the neuromuscular junction of the frog sartorius. Experiments on the ACh esterase concentration at the motor end plate following the section of the motor nerve have revealed that the high concentrations at this junction decreased by less than one-third within 3 to 4 weeks. The concentration then remains stable for several months. If, e.g., the ACh esterase concentration in a guinea pig gastrocnemius is determined in the way described above, in a muscle after the section of the motor nerve and the disappearance of nerve endings, the concentration of the enzyme in the middle zone remains

as high as before (Fig. 3). The results indicate that most of the enzyme at the motor end plate is apparently localized in the postsynaptic membrane, the site of the end plate potential, which is exclusively a muscular element (the sole plate of Kühne). From the data of Couteaux and Nachmansohn, it appears that less than one-third is localized in the presynaptic membrane. A similar distribution of the ACh esterase concentration between pre- and postsynaptic membrane may be assumed for the motor end plate of the frog on the basis of the data obtained by Feng and Ting (77). The amount which may be metabolized at one end plate of a frog sartorius during 1 millisecond would be able to cover a surface of  $300 \mu^2$  in the postsynaptic membrane and close to  $100 \mu^2$  in the presynaptic (calculated again on the basis of  $30 \text{ \AA}^2$  covered by one molecule of ester). These are potential rates. Experiments which will be described later indicate that the enzyme in the frog sciatic nerve is about 10 to 12 times in excess. But, the remaining 8 to 10% is still an impressive figure, especially if we keep in mind that only a fraction of the surface may be active during the passage of the impulse.

The experimental results concerning the distribution of ACh esterase in the sartorius have been confirmed by Feng and Ting (77). The conclusion has been contested by Clark, Raventós, Stedman, and Stedman (43) on the ground that the physiological concentration of acetylcholine is likely to be so low,  $10^{-6}$  to  $10^{-9}$ , that the enzyme will be acting at a very small fraction only of its maximum rate. There is, however, no indication what the physiological concentration of acetylcholine may be in the living cell. If the ester is metabolized, as it appears possible to assume on the basis of the enzyme distribution, in a few molecular layers, its concentration may be high. The difficulties encountered by the objections of Clark *et. al.* have been fully discussed (142). Little and Bennett (114), repeating the experiments with frog sartorius, confirmed also the existence of an uneven distribution of ACh esterase, but their differences were smaller than those described by Marnay and Nachmansohn (124). If acetylcholine metabolism is limited to the surface, it appears likely that the concentration of ACh esterase in a given fiber will depend on its size—the larger the fiber, the smaller the surface area per unit weight. Nachmansohn and Machado therefore tested the distribution of ACh esterase in the sartorius of frogs of different sizes (unpublished data). Table III summarizes the values obtained; the concentration in the pelvic end, free of nerve endings, is compared to that of the second fifth, rich in motor end plates, and the third fifth, with a smaller number of end plates. These observations may explain the difference between the data of Little and Bennett and the previous observations. The fact that the enzyme concentration in the pelvic end of a small-sized sartorius

is several times as high as in the fibers of the same muscle of larger size supports moreover the assumption that the enzyme is localized in the surface of the muscle fiber, as was shown previously for the nerve fiber. Since it is generally assumed that the mechanism of conduction is fundamentally the same in nerve and muscle, the enzyme may have the same function

TABLE III  
DISTRIBUTION OF ACh ESTERASE IN FROG SARTORIUS

Expt. no.	ACh hydrolyzed, mg./g. tissue/hour		
	Pelvic end	Second fifth	Third fifth
<b>A. Bullfrogs (sartorius weight 1.4 to 1.6 g.)</b>			
1	1.92	2.48	2.25
	1.25	1.82	1.49
	1.49	1.88	2.08
2	1.72	2.47	
<b>B. Medium-sized frogs (sartorius weight 60 to 100 mg.)</b>			
1	1.79	7.60	4.87
2	1.78	8.0	4.74
3	2.56	7.8	7.25
4	2.94	7.85	6.65
5	1.96	7.40	4.34
6	3.87	12.1	5.42
<b>C. Small frogs (sartorius weight 10 to 20 mg.)</b>			
1 (2) <sup>a</sup>	9.30	13.5	13.7
2 (2)	5.88	14.4	12.9
3 (3)	7.75	13.6	12.9
4 (3)	5.33	13.0	9.7
5 (1)	9.92	15.1	15.4
6 (3)	3.73	12.7	8.98
7 (3)	4.64	10.3	8.89
8 (2)	3.52	14.8	12.1

<sup>a</sup> Number of muscles per experiment.

there. Other observations to be described later are consistent with this assumption. In Table IV are given a few figures for the concentrations of ACh esterase found in muscles of various groups of animals.

#### D. SPECIFICITY

Esterases are widely distributed in the animal organism and are known to hydrolyze a great variety of esters. When a physiological function of acetylcholine associated with nerve activity was suggested, the important

question was, as pointed out by Stedman, Stedman, and Easson (195), whether there is a specific enzyme which has the physiological function to hydrolyze this ester. Stedman *et al.* prepared from horse serum an enzyme which they considered to be an esterase specific for choline esters and called it cholinesterase. Later investigations do not support the assumption that the enzyme prepared by Stedman *et al.* is really an esterase specific for choline esters. Vahlquist (200) was the first to show that the esterase in human plasma is not specific for choline esters, although these esters are split more rapidly than noncholine esters. In the extensive studies of Glick in which the hydrolysis of a great variety of esters by horse serum was tested, no evidence for specificity toward acetylcholine was found (84a).

During the last few years, however, the problem of the existence of an esterase specific for acetylcholine has attracted the attention of many in-

TABLE IV  
CONCENTRATION OF ACh ESTERASE IN MUSCLES OF VARIOUS ANIMALS

Species	Muscle	ACh split, mg./g./hr.
Guinea pig	Gastrocnemius	8-15
Fowl	Leg	4-6
Humming bird	Breast	80-100
Frog	Gastrocnemius	5-10
Lizard	Leg	30-40
<i>Lebistes</i>	Tail end	300-400
<i>Nereis</i>	Body wall	60-70
<i>Lumbricus</i>	Body wall	140-160

vestigators and has found a satisfactory answer. The new development started with the observations of Alles and Hawes (2) that red blood cell esterase differs markedly from serum esterase. They found that the esterase in the red cells has an optimum substrate concentration; further increase has an inhibitory effect. This is not the case with serum esterase. Acetyl- $\beta$ -methylcholine (mecholyl) is split by red cell esterase whereas it is not split by serum esterase (84a). Richter and Croft (183), confirming the work of Alles and Hawes, showed that red cell esterase is indeed highly specific for acetylcholine. In 1943, Zeller and Bissegger found that brain esterase is fundamentally similar to red cell esterase (205). The studies on the specificity of brain esterase toward acetylcholine as well as that of the esterase in all conductive tissues, nerve and muscle, were extended by Nachmansohn and Rothenberg (164,165). Testing a variety of esters on esterases of different tissues, they showed that the esterase in all conductive tissues and in erythrocytes has a number of well defined properties. The rate of hydrolysis of acetylcholine was found to be optimal at about 6 to

$8 \times 10^{-3} M$  substrate concentration. Higher concentrations inhibit increasingly the rate of hydrolysis. The enzyme splits propionylcholine at the same or at a lower rate than acetylcholine, whereas butyrylcholine was shown to be split at a low rate or not at all. The properties of a virtually pure ACh esterase obtained from the electric tissue of *Electrophorus electricus* do not differ from those found with other ACh esterases. The pattern obtained when the crude organ extract is used does not change during the course of purification, indicating that the esterase in the electric tissue is exclusively ACh esterase (148,188). In striking contrast to this type of esterase, it was found that the esterases of other tissues, or serum, split propionylcholine at a higher rate than acetylcholine, and butyrylcholine at a higher rate than propionylcholine. By these findings it became apparent that the use of propionyl- and butyrylcholine is essential for distinguishing the type of esterase present in conductive tissue from other enzymes splitting choline esters. In agreement with previous observations, Nachmansohn and Rothenberg did not find with serum and pancreas esterases a well defined optimum of acetylcholine concentration for the rate of hydrolysis. If the enzyme activity was plotted against the negative logarithm of the molar acetylcholine concentration, the usual dissociation curve was obtained, in contrast to the optimum observed with esterases of conductive tissue.

The observations of Augustinsson still further contributed to defining the specificity (9-12). He tested the effect of the change in substrate concentration ( $pS$ ) on the rate of hydrolysis, not only with acetylcholine but with a great variety of esters and enzyme preparations. With enzymes prepared from conductive tissue, he found that the optimum substrate concentration for propionylcholine and butyrylcholine usually coincides with that of acetylcholine, whereas other esters and especially noncholine esters, like triacetin, show different activity-substrate concentration relationships (13). Figs. 4 and 5 show the activity of four enzyme preparations as a function of substrate concentration. These four curves are selected to demonstrate the most striking similarity of the enzyme in all conductive tissues. In Fig. 4 are the results obtained with an unpurified and with a highly purified esterase from electric tissue of *Electrophorus electricus*. In Fig. 5 are given those with a solution prepared from the nucleus caudatus of ox and an extract from *Lebistes* muscle. The contrast to the other esterases is illustrated in Fig. 6, which shows the rate of hydrolysis of different esters at varying concentrations by a highly purified esterase of human serum. These observations have removed some contradictions and difficulties, especially the claim that the esterase of conductive tissue may split triacetin at a higher rate than acetylcholine and therefore may not be considered specific for acetylcholine. As may be seen in Figs. 4 and 5, this is apparently true at very high concentrations, where the acetylcholine

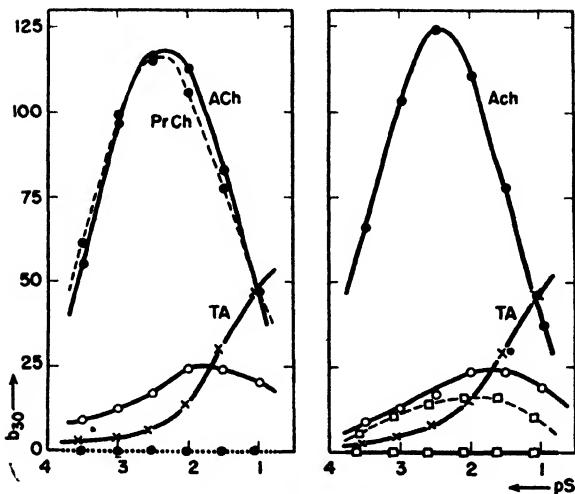


FIG. 4. Activity-pS curve of ACh esterase prepared from electric tissue of *Electrophorus electricus* (13). Left: crude extract, right: highly purified preparation (1 mg. protein splits 20 g. ester/hour). Abscissas: pS (negative log of molar concentration of substrate). Ordinates: Activity, mm.<sup>3</sup> carbon dioxide output/30 minutes. ●—● acetylcholine (ACh), ●---● propionylcholine (PrCh), ●....● butyrylcholine (BuCh), X—X triacetin (TA), and ○—○ acetyl- $\beta$ -methylcholine (mecholyl).

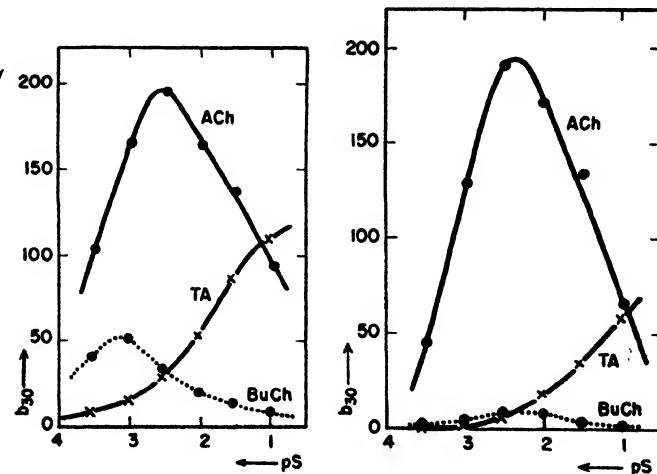


FIG. 5. Activity-pS curve of ACh esterase (13). Left: extract prepared from vertebrate muscle (*Lebiasa*). Right: extract from mammalian brain (nucleus caudatus of ox). Description and symbols as in Fig. 4.

already has an inhibitory effect on the enzyme, but actually the enzyme has a very low affinity for triacetin as compared to that for acetylcholine.

From all these investigations there emerged the fact that an esterase exists in all conductive tissues which has well defined properties and is highly specific for acetylcholine, although this specificity is relative and not absolute. Augustinsson and Nachmansohn have therefore proposed to call this enzyme acetylcholine esterase (ACh esterase) (15). Its most outstanding characteristics may be summarized as follows: The affinity of the

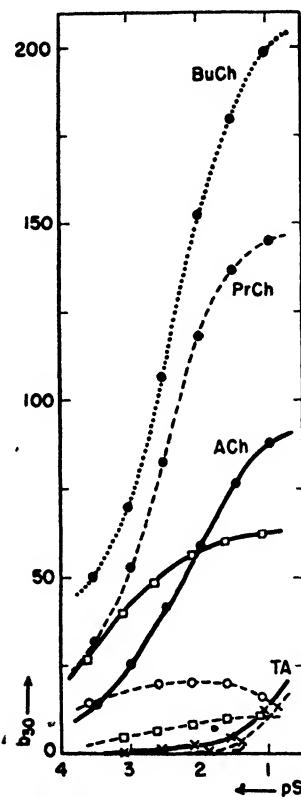


FIG. 6. Activity-pS curve of highly purified serum esterase (13). Description and symbols as in Fig. 4.

enzyme for acetylcholine is high; that means that the Michaelis constant is small. The turnover number is high; no other ester is hydrolyzed at a higher rate than acetylcholine. The enzyme splits propionylcholine at the same rate or more slowly, butyrylcholine at a low rate or not at all; non-choline esters are not split or split at a low rate, and have a very low affinity to the enzyme. If the enzyme activity is plotted against the logarithm of the molar concentration, a bell-shaped curve is obtained, consistent with the theory of Haldane (91) on inhibition of enzymes by substrates excess.

These properties make it possible to distinguish the ACh esterase from others. There are a few esterases, *e.g.*, in some sera, pancreas, and presumably in other tissues, which split choline esters at a higher rate than noncholine esters. In agreement with the usual enzymological terminology, these esterases may be called cholinesterases, as was originally proposed by Stedman for the serum esterase. Their physiological substrate is not known. Therefore the name appears, at least at present, appropriate. These esterases have a relatively high Michaelis constant for choline esters and do not show a well defined optimum if the hydrolysis rate is tested as function of pS. The rate of hydrolysis increases with increasing length of the acyl chain from two to four carbon atoms. This type of esterase differs also from the large group of those which split noncholine esters at a higher rate than choline esters and which at present cannot be classified at all.

The erythrocyte esterase shows the same properties as that in conductive tissue. A function of acetylcholine in red blood cells is unknown but it may be recalled that Brauer and Root (28) found the enzyme localized in the surface of the red cells. It is, moreover, known that the surface of erythrocytes has certain similarities with the nerve cell membrane. If, as will be discussed later, acetylcholine metabolism is important for the permeability changes occurring during nerve activity, it is not inconceivable that the ester has a similar function in the erythrocyte membrane. This possibility is apparently being explored by Greig and Holland (88a).

There are, in addition, two special cases in which the esterase has been found to be similar to that of conductive tissue: in the blood of *Helix pomatia* (12), and in snake venom (13,204). The latter finding is not surprising. Snake venom is a fluid containing a great variety of enzymes. The presence of the enzyme in the blood of *Helix pomatia* is more difficult to explain. It may be noted, however, that Augustinsson (12) has found deviations in several respects of the properties of this enzyme from the typical properties of the ACh esterase.

Considerable confusion has been created by the introduction of the terms "true" and "pseudo" cholinesterase by Mendel and associates. In contrast to other investigators, Mendel and Rudney (126) found the optimum rate of acetylcholine by brain esterase to be below 0.0002 M substrate concentration. They considered this optimum rate at an extremely low concentration to be the decisive difference of the "true" as compared to the "pseudo" cholinesterase, for which they found an optimum of above 0.02 M. As outlined by Nachmansohn and Rothenberg, these data were based on inadequate technique (165). Mendel and Rudney later claimed that the low concentration is the optimum for the rate of hydrolysis when distilled water is used as medium, whereas in presence of a high potassium concentration the optimum rate shifts to higher substrate concentration

(127). This claim could not be confirmed by Augustinsson (12). The expressions "true" and "pseudo" have been generally opposed by enzyme chemists. As Glick (84b) stated: "the undesirability of the term is apparent and it should be dropped from the literature." Glick's advice appears all the more justified since the facts on which this terminology is based have not been confirmed.

The free energy change of acetylcholine hydrolysis has been determined by Hestrin (91b). With the use of the highly purified preparation of acetylcholine esterase obtained from the electric tissue of *Electrophorus electricus* he demonstrated an equilibrium:

$$\frac{[\text{acetylcholine}^+][\text{water}]}{[\text{acetic acid}][\text{choline}^+]} = K$$

The Nernst equilibrium constant (K) calculated by using molarities rather than activities, was found to have the value 0.25 at 23°C. The free energy change of the ester hydrolysis,  $\Delta F$ , may be estimated from K by the equation:

$$\Delta F = -RT \ln (55.5/K)$$

whose derivation has been discussed by Meyerhof and Green (129a).  $\Delta F$  calculated in this way was found to be approximately -3100 cals. An equilibrium of a similar form was observed in the presence of propionic acid.

Neurath and his associates have recently demonstrated the ability of proteolytic enzymes to catalyze ester hydrolyses (99a). It is, therefore, of interest that acetylcholine esterase has been found to catalyze not only O-acylations but also N-acylations [Hestrin (91c)]. When sodium acetate or propionate was incubated with hydroxylamine in the presence of purified acetylcholine esterase obtained from electric tissue of *Electrophorus electricus* formation of hydroxamic acid was obtained. The substrate concentrations required for these reactions are high, compared to the optimum concentration of acetylcholine. This indicates that the affinity of the enzyme for acetylcholine is of a much higher order of magnitude. The substrate concentration-activity relationship observed in the acylation of hydroxylamine resembles that of neutral ester hydrolysis by acetylcholine esterase (165,13).

If inhibitors of the enzyme are present in the reaction mixture, e.g. prostigmine or tetraethylpyrophosphate, the acylation can be slowed or entirely prevented.

#### E. UBIQUITY

From the physiological point of view, the presence of a special enzyme with a high affinity for acetylcholine in all conductive tissue appears to be a most significant fact. ACh esterase is found in all types of nerves,

invertebrate and vertebrate, motor and sensory, central and peripheral, cholinergic and adrenergic, and in muscle. The esterase occurs in *Tubularia*, the lowest hydrozoan to possess neuromuscular tissue comparable to that of higher animals (34).

The presence of a specific enzyme in a tissue may be used as an indicator for the metabolism of the substrate. One of the most important general results of the work with isotopes is the conclusion that enzymes "do not lie dormant during life, but are continuously active" (191). This does not imply that all enzymes are working continuously at an optimal rate. Enzymes are usually far in excess of the optimum required and, in addition, in cells like nerve and muscle, a considerable difference must be expected between resting condition and the state of activity. But, on the basis of all experiments in enzyme chemistry, it appears justified to assume that the presence of an enzyme in a cell indicates that the substrate is metabolized there and that a certain correlation exists between the concentration of the enzyme and the rate of metabolism. This of course is only true in the case of a specific enzyme, and, for this reason, it was important to demonstrate the specificity of ACh esterase. Its presence in all conductive tissue becomes particularly significant in connection with the observation that its inactivation blocks conduction in all these tissues, as will be described later.

#### F. CONCENTRATION OF ACETYLCHOLINE ESTERASE AND FUNCTION DURING GROWTH

A correlation between ACh esterase concentration and function has been observed in experiments on embryonic tissues. It has been demonstrated in a variety of cases that, during growth, the high concentration in nerve tissue and at motor end plates is reached at the period when function develops. The first such observation was made on the muscle of the chick embryo (140,141). Usually, the enzyme concentration in growing tissue increases progressively until it has reached a maximum, frequently in an S-shaped curve. In contrast to this usual pattern, it was found that the ACh esterase concentration in the muscle of the chick embryo rises to high values around the twelfth to fourteenth day of incubation, i.e., the time when the muscular movements begin. The concentration then increases slightly but, after hatching, the values begin slowly to fall, and 3 weeks later they are less than 10% of those at hatching (Fig. 7). At hatching, the muscle fibers are small, and therefore the end plates per unit weight of muscle are relatively numerous. Later, the fiber grows, and the number of end plates per unit weight decreases. The fact that the concentration of ACh esterase is so high at the early stages of development, when the number of end plates is high per unit tissue weight, suggests that the concentration of ACh esterase at the end plate is high at that early period.

There are a number of other observations which show the coincidence between the appearance of the high concentration of ACh esterase and the beginning of function. In the central nervous system the time when the centers begin to function coincides also with the time when the high concentration of the enzyme appears (139,142). In the brain of the chick embryo, the  $Q_{ACh.E.}^1$  rises from 100 at the sixteenth day of incubation to 200 at hatching; during the first week after hatching, it increases further to 250 to 260, which is the same value as in fowl brain. The functions of chicken brain are fairly well developed at hatching. The rapid rise of ACh esterase

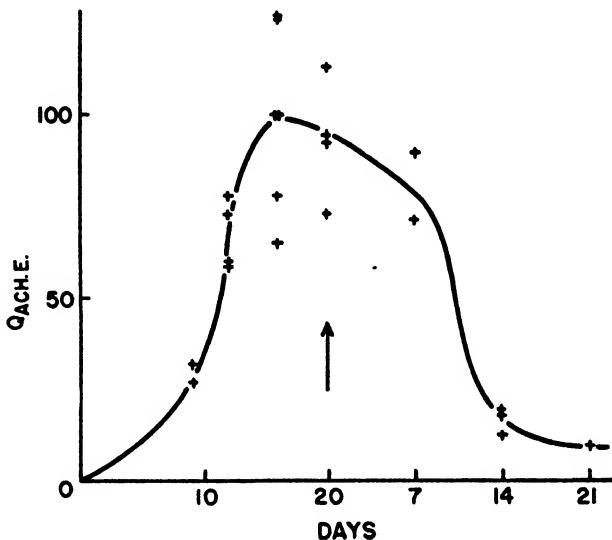


FIG. 7. Changes of the concentration of ACh esterase in breast muscle of chicken. Abscissae: Days of incubation and after hatching. Arrow marks day of hatching.

during the last 4 days of incubation is particularly striking when compared to the figures in the brain of young mammals. In the brains of newborn rats and rabbits which are markedly undeveloped, the enzyme concentration is very low; during the first 3 weeks after birth, the concentration increases rapidly to high values and that is the time during which the brain functions develop. In the brain of newborn guinea pigs, on the other hand, which are well developed at birth, the enzyme concentration is nearly as high as in adults.

The different centers of the central nervous system do not develop at an equal rate. During recent years this problem has been investigated by Barcroft and Barron (20) in connection with the movements and reflexes

<sup>1</sup>  $Q_{ACh.E.}$  is defined as milligrams acetylcholine split per gram fresh weight tissue in 60 minutes.

of sheep fetuses. Their observations offer evidence for the early development of spinal reflexes and of the relatively delayed period at which the brain enters into action. At first there are local reflexes, later the reticulospinal system becomes more and more important, and only at a late period the brain takes over the "dominance of the body." The time when the different centers begin to function coincides again with the appearance of a high concentration of ACh esterase. This concentration is high in the spinal cord at a very early age of the sheep fetus, but low at that time in the different brain centers, where it rises to high values only during the

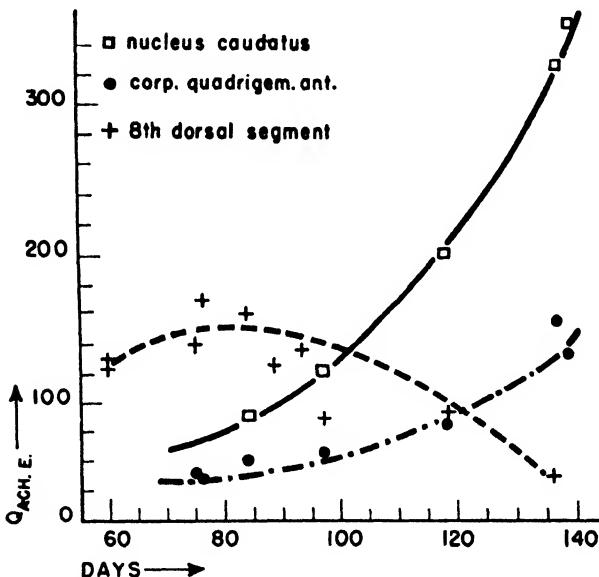


FIG. 8. Concentration of ACh esterase in brain and spinal cord of sheep fetus during growth. Abscissae: Days of gestation time.

last weeks before birth (144). Some of the observations are reproduced in Fig. 8. Similar observations carried out on the human fetus led to the same result (203). Here again, a significant relationship was found between ACh esterase concentration in some brain centers and developing motility and behavior.

An interesting contribution to this problem is the study of Sawyer (190) on the ACh esterase concentration during the development of the larval life of *Amblystoma*. He found significant correlations to exist between the concentration of the enzyme present and the functional ability as expressed by behavior manifestations. The enzyme concentration is low during the early stages of development but rises sharply with the onset of the S-flexure and the more rapid movements during the swimming reactions. The

functional capacity of the larvae reared in solutions of eserine is profoundly affected. On removal of the animal from the inhibitor solution, the recovery of physiological capacity and enzymic activity parallel each other.

To summarize, the following features of ACh esterase have been described so far: the speed of the enzymatic reaction, which parallels that of the electrical manifestations; the high concentration of the enzyme in nerve and muscle tissue; the exclusive localization in the axonal surface; the specificity of the esterase for acetylcholine in conductive tissue in contrast to other esterases, and its ubiquity; and finally, the coincidence of the beginning of function with the time when the high concentration appears during growth. Every one of these features is consistent with the assumption that the activity of ACh esterase is important for conduction, and, considered altogether, they appear suggestive. However, for demonstrating conclusively the necessity of the enzyme for conduction, the enzyme activity must be correlated with the electrical manifestations. This has been achieved in several ways.

### III. Correlation between Electrical Events and Chemical Reactions in Electric Tissue

#### A. PARALLELISM BETWEEN VOLTAGE OF ACTION POTENTIAL AND ACTIVITY OF ACETYLCHOLINE ESTERASE

The first such correlation was established in experiments on electric fish. That the powerful shock of these fish, known since ancient times, is an electric discharge was demonstrated by Welsh before the Royal Society in London in 1772. It was the first demonstration of animal electricity, and attracted the attention of many physicists, like Cavendish, Michael Faraday, Sir Humphrey Davy, and others. Following Galvani's observations, physiologists became interested in these fish. Galvani himself in the last year of his life worked on electric fish, and during the last century other physiologists, especially Du Bois Reymond, investigated different aspects of the electric discharge in these fish.

The most important feature of electric organs is the generally accepted fact that the nature of their activity is identical with the action current of nerve and muscle. The potential difference developed by a single physiological unit, the electric plate, is about 0.1 v., which is of the same order of magnitude as that found in ordinary nerves. It is only the arrangement of these plates in series by which these organs are distinguished. The great differences of the total discharge in various species do not depend on the units, which show only relatively small variations, but on the shape and dimensions of these species. In the species with the most powerful electric organ known, *Electrophorus electricus* Linnaeus, found in the Amazon river, several thousand plates are arranged in series from the head to the caudal

end of the organ. Thus, the voltage of a discharge is on the average between 400 and 600 v., and in some specimens may be even higher. In *Torpedo*, another genus with a powerful electric organ, the elements are arranged in a dorsal ventral direction. Since it is a flat fish, the number of plates in series is much smaller. In the *Torpedo marmorata*, a species found on the west coast of France, the number does not exceed 400 to 500 and, consequently, the discharge is on the average 40 to 50 v. A particularly large species of *Torpedo* can be found occasionally in the water around Cape Cod; the *Gymnotorpedo occidentalis*, first described by Storer in 1848. In this species, 150 to 200 v. was measured by Amberson and Edwards (see 163). All the typical features of the discharge are the same as known from the action potential in neurophysiology; latency, duration of the discharge, and the refractory period are of the same order of magnitude as in the nerve action potential. A full description of earlier work can be found in many handbooks and textbooks (185). More recent investigations on the electrical aspects were carried out by Auger and Fessard (6-8), Cox, Coates, and associates (44,45,55,56) and Chagas (40,41). Important studies of the electrical properties of a few isolated units have been carried out in a most elegant way by Auger and Fessard (6-8).

Recent developments have led to a more precise identification of the discharge of the electric tissue. As was shown by Babuchin in 1870, the electric organs have phylogenetically evolved from striated muscle. The only exception is possibly the organ of *Malapterurus*, the origin of which is doubtful. In strong electric organs, the contractile elements have completely disappeared but they exist as rudiments in the plates of weak electric organs. The electric plates are therefore more exactly homologous to the motor end plates. As has been described by several authors in the last century, the electrolemma surrounding the nerve endings in the electric organ shows, on the side toward the cell interior, a very remarkable structure, a layer of rods, the "palisades" of Remak. Recently, it has been shown by Couteaux (50,57) that the postsynaptic membrane at the motor end plate has interesting similarities with the postsynaptic membrane surrounding the nerve endings in the electric tissue. By using Janus green or methyl violet, he was able to demonstrate the existence of a very peculiar structure of the sarclemma surrounding the nerve endings but separated from them by a layer of neuroglia (called "teloglia" by Couteaux).

On the other hand, the observations of Gopfert and Schaefer (86) and the work of Eccles and associates (68) have revealed the existence of a special end plate potential generated in the postsynaptic membrane. Since the electric plates are homologous to the motor end plate, the discharge in electric tissue must be considered more precisely as homologous to the end plate potential. This view finds strong support in the remarkable similarities of the morphological correlate of these two potentials.

Krogh (102b) once stated that nature has 'created' quite a number of animals for special physiological problems. The electric tissue appears indeed to be a most suitable material for correlating chemical reactions with electrical manifestations. In ordinary nerves, the electrical processes are on such a small scale and associated with such small amounts of energy that the available methods are inadequate for studying the underlying chemical reactions. In 1937, the electric tissue was introduced by Nachmansohn as material for the study of the role of acetylcholine in the mechanism of nerve activity, especially in view of the homology with the neuromuscular junction.

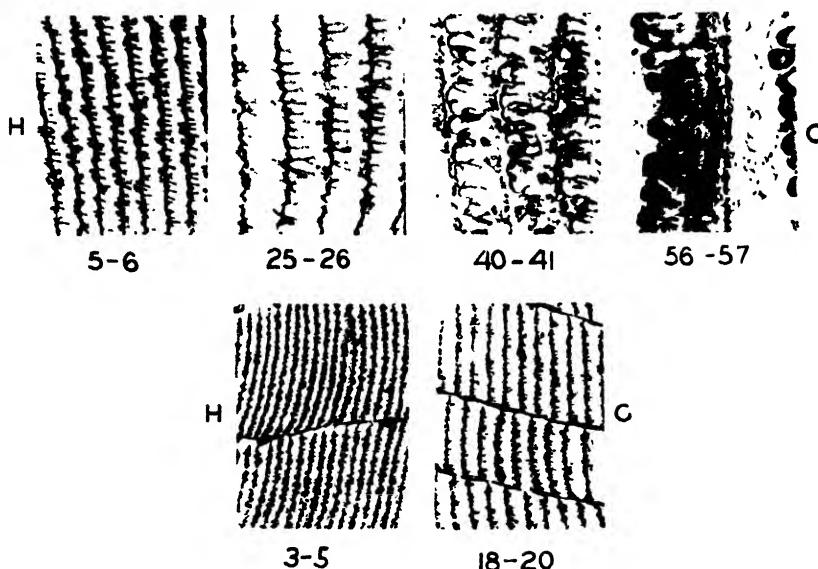


FIG. 9 Histological aspect of electric tissue at different sections of the organ ( $\times 145$ ). Specimen used for upper sections 114 cm. long, that for lower sections 57 cm. long. Numbers indicate distance in cm. from the anterior end of organ H, head end, C, caudal end.

Marnay, on his suggestion, determined the ACh esterase in the electric organ of *Torpedo marmorata* and found that 1 g. electric organ splits 2.0 to 3.0 g. acetylcholine in 60 minutes (121). In the electric organ of *Electrophorus electricus*, the concentration is also high, whereas it is low in the weak electric organs of the common ray (145,163). The existence of the extraordinarily high concentration in electric tissue appears particularly significant in view of the high water and low protein content of these organs. 92% is water and only slightly more than 2% protein.

A close relationship exists between the concentration of ACh esterase and the voltage and number of plates/cm. The electric organ of *Electro-*

*phorus electricus* is particularly favorable for the demonstration of this relationship. The number of electric plates/cm. varies considerably with the size of the specimen and decreases, moreover, markedly from the head to the caudal end of the organ in each specimen. These variations are illustrated in Fig. 9. Since each plate develops, as mentioned above, about the same voltage, the voltage/cm. varies correspondingly. A close parallelism was found between the number of electric plates, the voltage/cm., and

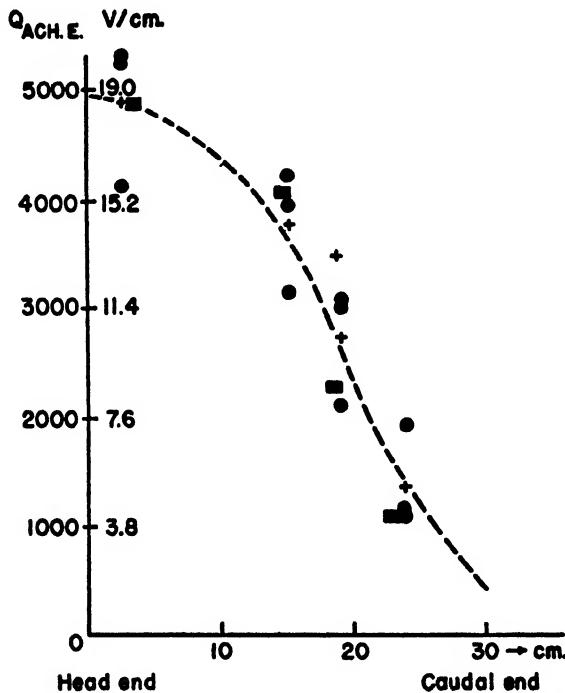


FIG. 10. Parallelism between action potential and ACh esterase concentration in the electric tissue of *Electrophorus electricus*. Length of specimen, 51 cm. ● average  $Q_{ACh.E.}$  from a single piece of tissue, + average  $Q_{ACh.E.}$  from pieces of the same section, ■ v./cm.

the concentration of ACh esterase, as may be seen in Fig. 10 (155). A great number of experiments has been carried out on fish of various sizes covering a range of the action potential from 0.5 to 22 v./cm. Thus, a statistical evaluation of the relationship between voltage and ACh esterase activity became possible. The average quotient—ACh esterase concentration over voltage—was found to be 207 with a standard deviation of only  $\pm 7$  or 3.7%. The standard deviation for a single measurement is  $\pm 51$  or about 25%. This is good uniformity for a quotient correlating

physical and chemical data. If the voltage/cm. is plotted against the ACh esterase concentration, the resulting line calculated by the method of least squares goes virtually through 0, which indicates a direct proportionality between voltage/cm. and ACh esterase activity (Fig. 11) (154). The direct proportionality between physical and chemical events suggests the interdependence of the two events.

The finding is significant in view of the changing morphological structure of the electric unit, the electric plate. If all plates were identical in structure as, e.g., in the case of the electric tissue of *Torpedo*, the voltage and ACh esterase concentration could be expected to be directly proportional

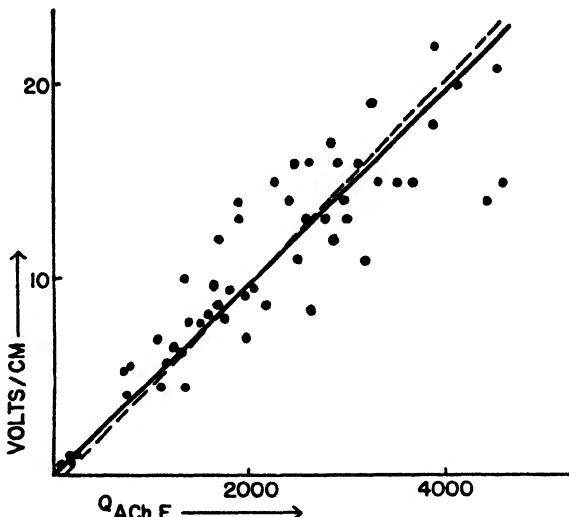


FIG. 11. Correlation between voltage and ACh esterase concentration. Dotted line calculated from data obtained with method of least squares; solid line calculated on assumption that line goes through zero point.

to the number of plates, since it is known that the enzyme is localized in the surface. The situation is entirely different in the electric organ of *Electrophorus electricus* because the structure of the plates shows enormous variations. In spite of all variations of the visible structure, the voltage of each plate is the same, *viz.*, close to 100 mv. It has therefore to be assumed that the active membrane with which the electrical manifestations are connected and which is not yet well defined does not change, but is similar in all plates. The direct proportionality found between voltage and ACh esterase suggests, then, that the physical and chemical events may be associated with the same membrane and that they may be functionally interdependent. The great variations of ACh esterase are in striking contrast to the distribution of other enzymes and compounds, like

adenosinetriphosphatase (ATPase), respiratory and glycolytic enzymes, phosphorylated substances, etc., which do not show significant variation in concentration in relation to the voltage developed. This is of particular interest in the case of ATPase, which has been reported to be localized almost exclusively in the surface of the giant axon, as is ACh esterase (106).

### B. CHEMICAL REACTIONS SUPPLYING ENERGY FOR THE ACTION POTENTIAL

The changes in the neuron surface during activity which are associated with the release of electric energy and their rapid reversal cannot conceivably be effected without energy loss, since they must involve processes which are irreversible from the thermodynamic point of view. Obviously, chemical reactions must supply the energy lost during the discharge. The electric tissue, in view of the magnitude of the electrical events, appeared again to be the best material for an analysis of the chemical reactions which may supply the energy.

The most readily available source of energy for endergonic life processes is the energy of phosphorylated compounds rich in energy, as has been established by the work of Meyerhof and his school and extended by the investigations of Parnas, Cori and Cori, D. M. Needham, and others. In muscle, the breakdown of adenosinetriphosphate (adenylpyrophosphate, ATP) appears to be the primary energy source for the contraction (128, 129). The ATP formed is rephosphorylated by the breakdown of phosphocreatine, a phosphate shift which occurs without loss of energy. Phosphocreatine thus acts as a storehouse for energy-rich phosphate.

The presence of phosphocreatine in the electric organ was first described by Kisch (102), who suspected that this compound might be connected with the activity of the organ. More intensive studies of the enzymatic reactions in extracts of electric organs, especially those connected with the phosphorylated compounds, were carried out by Baldwin and Needham (19). These investigators showed that the main steps known to occur in intermediate metabolism of muscle also occur in those extracts. These experiments were carried out on *Torpedo*. In the electric tissue of *Electrophorus electricus*, the phosphocreatine concentration is high—as high as or even higher than in striated muscle, in spite of the low protein and high water content. The concentration of ATP, although lower than in muscle, is also marked (156).

Measurements carried out on the electric organ of *Electrophorus electricus* have revealed that the chemical energy released by the breakdown of phosphocreatine is adequate to account for the electrical energy released by the action potential. Both chemical and electrical energy per gram and impulse vary considerably depending on the size of the specimen—the

larger the size, the smaller is the rate of metabolism. The total electrical energy released per gram and impulse in large eels of 170 to 180 cm. length was found to be about 24 microcalories. The electrical energy released externally is, under the most favorable conditions, only one-sixth of the total. But for the relationship between electrical and chemical energy released, only the total has to be considered. In medium-sized specimens of 90 to 120 cm. length, the total electrical energy per gram and impulse was found to be about 47 microcalories. These figures are based on some assumptions which have been discussed by Cox, Coates, and Brown (56). If all probable assumptions are considered, the values may possibly have to be revised, either downward by 15% or upward to 100%.

TABLE V  
RELATIONSHIP BETWEEN ELECTRICAL ENERGY RELEASED BY ACTION  
POTENTIAL AND CHEMICAL ENERGY RELEASED BY BREAKDOWN OF  
PHOSPHOCREATINE AND FORMATION OF LACTIC ACID

Length of fish, cm.	Energy released/g./impulse, g. cal. $\times 10^{-6}$				Electrical energy, % of chemical
	Electrical <sup>a</sup>	Phosphocreatine	Lactic acid	Combined	
103	47 (9)	54.5 (21)	68.5 (21)	123.0 (21)	38
180	24 (6)	35.7 (15)	23.1 (7)	53.4 (7)	45

<sup>a</sup> Average values. Figures in parentheses indicate number of experiments on which values are based.

Tested under the same conditions, the energy released per gram and impulse by the breakdown of phosphocreatine has been found to be higher than the total electrical energy in both large- and medium-sized specimens (153). Figures obtained with *Electrophorus electricus* are given in Table V. But, in addition, there is energy released by the simultaneous formation of lactic acid. It may be assumed that the energy of the lactic acid formation is used, as in muscle, to rephosphorylate creatine by the Parnas reaction, i.e., the phosphopyruvic acid transfers its phosphate to creatine using adenosinediphosphate (ADP) as intermediate. The sum of the two reactions may therefore be used as indication of the energy supplied by energy-rich phosphate. The figures are calculated on the assumption that 11,000 kcal./mole are released by the breakdown of phosphocreatine and 22,000 kcal./mole of lactic acid formed (153). The energy released by the two reactions is adequate to account for the total electrical energy released even if this is twice as high as estimated—and that would be, on the basis of all probable assumptions, the highest figure. Taking the most likely assumption, the electrical energy would be about 40% of the chemical energy

released by the two anaerobic reactions measured. For evaluating the efficiency of the process, the energy supplied by aerobic recovery has to be added. This energy will probably be higher than the two anaerobic reactions combined.

Although the extra oxygen uptake following the activity has not been measured, it is of interest to evaluate, on the basis of the available figures, the relationship between the respiration and the energy released by the energy-rich phosphate.  $7.7 \times 10^{-6}$  moles phosphocreatine are split per gram tissue per 1600 impulses in the medium-sized eels. The  $Q_{O_2}$  of slices of electric tissue of *Electrophorus electricus* is low, on the average about  $-0.4$ , which is the same order of magnitude as in most peripheral-nerve tissue. This means that  $1.5 \times 10^{-5}$  mole oxygen/g./hour is used. According to Ochoa (175), in heart muscle three energy-rich phosphates are produced for each atom of oxygen used. In electric tissue this ratio has not been measured but it is reasonable to assume that it is of the same order of magnitude. The regeneration of the energy lost by the phosphocreatine split by 1600 impulses would then require about  $1.3 \times 10^{-6}$  mole oxygen, or less than 10% of the oxygen uptake per hour measured in slices. To the phosphocreatine breakdown has to be added that of lactic acid formation. The two reactions together yield, per gram electric tissue and per 1600 impulses, about 0.2 gcal. 1 g. electric tissue takes up 320 mm.<sup>3</sup> oxygen in 60 minutes (on the basis of a  $Q_{O_2} = -0.4$ ). Assuming  $4.8 \times 10^{-5}$  gcal./mm.<sup>3</sup> oxygen metabolized, the oxygen used per hour would yield 1.5 gcal. The efficiency of the conversion of oxidation energy to phosphate energy is close to 60% (175). Thus, 0.33 gcal., or about one-fifth the respiration energy per hour measured with the slice technique, would be required to account for the energy lost by the phosphocreatine breakdown and the lactic acid formation after 1600 impulses. The rate of respiration in slices may be higher than in the resting cell *in situ*, although it appears unlikely that it is as high as following maximum activity. But the figures indicate that a moderate increase of respiration above that in resting state may well be sufficient to account for the energy lost by 1600 impulses. After this number of impulses there are generally no signs of fatigue. Usually, the number of discharges is between 3000 and 8000 when signs of marked fatigue appear. In ordinary nerves, the extra oxygen uptake following prolonged activity may rise to twice or three times the resting value. It appears possible to assume from the figures obtained that a rise of a similar order of magnitude may occur in the electric tissue following maximum activity.

As mentioned above, the work of Meyerhof and his school has shown that the breakdown of ATP precedes that of phosphocreatine. It is today believed, on the basis of the work of Engelhardt and Ljubimova, Needham, Szent-Györgyi, and their associates, that ATP may react directly with the muscle protein and that this may be the primary chemical reaction in muscular contraction. It is safe to assume, therefore, that the breakdown of ATP occurs during nerve activity as in muscle, before that of phosphocreatine. But it appeared unlikely, for many reasons, that ATP breakdown is the primary reaction connected with conduction as recently proposed (106), because of the marked differences between conduction and contraction. Among other obstacles, the greatest difficulty is the time factor.

There is no evidence that ATP breakdown may occur at the speed required for the primary event in conduction. The turnover number of ATPase is 8000/minute as compared to 20,000,000 for ACh esterase. On the basis of the available evidence, it appeared more likely that the release and removal of acetylcholine are the reactions more directly connected with the alterations of the active surface membrane during the passage of the impulse and that they occur prior to the breakdown of ATP. The latter would then be the primary recovery process supplying the energy for the resynthesis of the acetylcholine hydrolyzed during activity.

It appeared crucial, then, to test whether or not the energy of ATP provides the energy of acetylcholine formation. If this is the case, it would show that the energy of the primary recovery process is really used for the resynthesis of the compound, the release of which is supposed to be directly associated with conduction. A new enzyme, choline acetylase, has been extracted from brain which in cell-free solution under strictly anaerobic conditions forms acetylcholine in the presence of ATP (162). The discovery of this enzyme system constitutes support for the assumption that the breakdown of acetylcholine precedes that of ATP, the latter being the primary recovery process. It was the first demonstration that acetylation in living cells can derive its energy from energy-rich phosphate and, more generally, at that time (in 1943) the first demonstration that the energy of ATP may be used outside the glycolytic cycle.

Thus, the observations led to an integration of acetylcholine into the metabolic cycle. Fig. 12 illustrates how the sequence of reactions connected with the passage of the nerve impulse may be pictured. The formation of acetylcholine is, as will be seen later, a relatively slow process compared to its hydrolysis. It appears therefore likely that this reaction occurs in recovery. The ester must be assumed to be present in an inactive form bound to protein or lipoprotein and protected against the action of ACh esterase. If ions, potassium or perhaps sodium, activated by the flow of current, hit this acetylcholine complex, the ester is released. Having produced its effect, the free ester is hydrolyzed by ACh esterase into choline and acetate, both parts of the molecule being inactive compounds. Thus, the action of the free ester is stopped and the restoration of the membrane to its original condition initiated. In the recovery period, choline acetylase, using the energy-rich phosphate of ATP for the synthesis, and in presence of potassium and magnesium ions and a coenzyme (see next section), resynthesizes acetylcholine. The other steps are, as indicated in the figure, the same as known from the metabolic cycle in muscular contraction. Fundamentally, the two cycles, *i.e.*, that of conduction and of contraction are the same, except for the first reactions. In view of the basic difference between these two functions, this is not surpris-

ing. The energy wheel driving the process of contraction must be much more powerful but may be slower than that driving the process of conduction. In the series of successive metabolic energy wheels, that of acetylcholine is by far the fastest, but has less energy than the others, and may be closest to the protein or lipoprotein change associated with the activity of nerve. The slower the energy wheel, the farther away it will be from the actual change during activity and the ultimate source of energy in both cases is the slow but powerful wheel of respiration.

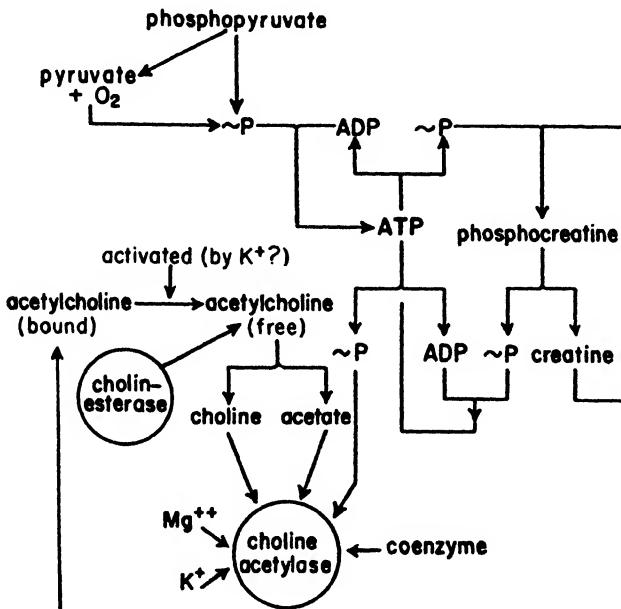


FIG. 12. Integration of hydrolysis and formation of acetylcholine into the metabolic cycle of nerve activity.  $\sim P$  denotes high-energy phosphate.

In this connection it is of interest to compare the low rate of respiration to the high rate of acetylcholine metabolism. If the tissue splits 2 to 4 g. acetylcholine/g./hour, about 10,000 to 20,000 times more molecules of acetylcholine can be split per unit tissue and unit time than molecules of oxygen taken up. If the oxygen uptake after activity is increased to twice the observed value, the rate of hydrolysis would still be 5000 to 10,000 times as high as that of respiration. However, these figures do not indicate the real ratio between the possible rate of acetylcholine hydrolysis and that of oxidation since they are calculated per unit tissue weight. Acetylcholine metabolism, however, occurs only in the neuron surface, *i.e.*, within a minute fraction of the actual tissue weight. In contrast, oxidation occurs

throughout the axoplasm (169,170). If the ACh esterase is contained in 5% of the tissue, the rate of hydrolysis would be 200,000 to 400,000 times as high as respiration, but since the assumption of 5% for the active membrane is probably much too high, the difference of the two rates of metabolism is probably higher than one million times. A sharp distinction has, however, to be made between the possible rate and the absolute amounts actually metabolized. Acetylcholine may be released and hydrolyzed within milliseconds. The duration of the discharge is about 2 to 3 milliseconds. The number of the discharges until fatigue is only a few thousand. The whole duration, *e.g.*, of 6000 discharges, would then be about 15 to 18 seconds. The complete recovery requires more than an hour; the increased rate of respiration may continue for 1 or 2 hours. Thus, the difference between the amounts actually metabolized becomes small and as we have seen, if the energy released by the different reactions is evaluated, a satisfactory picture is obtained. It may be noted that the energy released by phosphocreatine or ATP is about 11,000 kcal. while the acetylation of choline requires not more than 3000 kcal. Since there is some evidence that the reaction may be stoichiometric (171), the fact of the remaining energy has to be explained. It is possible that it is used for the formation of the complex but, so far, there is no experimental evidence for this or any other simultaneous reaction. There are, of course, several processes conceivable which could account for the energy released by the energy of the phosphate and not used for the acetylation of choline.

#### IV. Choline Acetylase

##### A. PREPARATION OF THE ENZYME

Synthesis of acetylcholine *in vitro* was first observed by Quastel and associates (120,180). These investigators found an increased formation of acetylcholine in brain slices under anaerobic conditions. The elucidation of the enzymatic mechanism became possible only following the discovery that ATP is the energy source for the synthesis and by the extraction of the enzyme system from the cell (162). Working first with minced or homogenized tissue, Nachmansohn and Machado found only a small synthesis of acetylcholine, or none at all. But when they prepared cell-free extracts, a high rate of formation, up to 150 µg. acetylcholine/g./hour (fresh rat brain) was obtained. In addition to ATP as energy source, and choline and acetate as substrates, fluoride and eserine must be present in order to inhibit the action of ATPase and ACh esterase, respectively. Ochoa has shown that fluoride inhibits the activity of ATPase without interfering with the transfer of phosphate to a phosphate acceptor (174). This may be due to the precipitation of calcium, since it is known that calcium activates ATPase (18). In fresh brain extracts, fluoride greatly increases the rate of acetylcholine formation.

Extractions prepared from fresh tissue are very unstable and the rate of formation decreases rapidly. When, however, extracts were prepared from acetone-dried powder, a much more stable solution was obtained (158,159). Moreover, in this way, choline acetylase is separated from most of the ACh esterase and all of the ATPase, since treatment with acetylcholine inactivates these two enzymes. In the first preparations, less than 1 mg. acetylcholine was formed/g. powder/hour. Later, in the more complete system, the values obtained were 2 to 3 mg./g. powder/hour. The findings of Nachmansohn and associates have been confirmed in several laboratories (108,112,194).

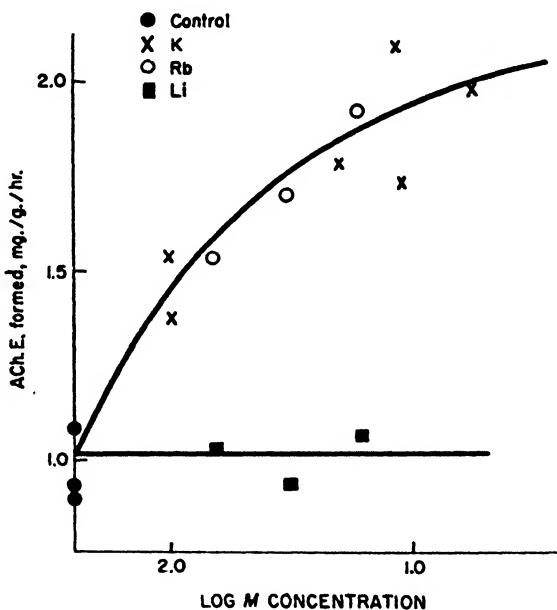


FIG. 13. Effect of potassium, rubidium, and lithium on choline acetylase prepared from acetone-dried powder of rabbit brain.

#### B. PURIFICATION OF THE ENZYME AND CONDITIONS OF OPTIMAL ACTIVITY

The formation of acetylcholine by the choline acetylating system requires for optimal activity the presence of potassium, magnesium and calcium ions (150,159,171a). The potassium may be replaced by rubidium. Sodium and lithium ions have no effect. Fig. 13 (171).

The significance of sulphydryl groups present in so many enzyme proteins has been stressed by the work of Barron (21). Choline acetylase belongs to the class of enzymes containing sulphydryl groups which are easily oxidized. The formation of acetylcholine is strongly inhibited by iodoacetic

acid, which, as shown by Rapkine (181,182) and Dickens (63), reacts with sulfhydryl groups. In  $10^{-3} M$  concentration, iodoacetate completely inactivated choline acetylase within 20 minutes (162). Iodine, which readily oxidizes sulfhydryl groups, also has a marked inhibitory effect on the enzyme in  $10^{-4} M$  concentration if allowed to act for 40 to 60 minutes. Sulfhydryl groups are highly sensitive to copper, although this effect is not entirely specific. Formation of acetylcholine in fresh extracts was virtually completely inhibited by  $3 \times 10^{-5} M$  concentration of copper ions and the inhibition was still 66% at  $1.5 \times 10^{-5} M$  concentration. Further support for the high sensitivity of the sulfhydryl groups of choline acetylase may be seen in the fact that, in fresh extracts, the yield is considerably decreased by incubation in air instead of under strictly anaerobic conditions (159). Presence of cysteine improves the yield considerably and, in sufficiently high concentrations, may prevent any effect of oxygen. Even under anaerobic conditions, the presence of cysteine may increase the rate of formation in fresh extracts. In extracts which have been dialyzed or prepared from acetone-dried powder, the presence of cysteine in high concentrations becomes necessary, since these treatments apparently inactivate the sulfhydryl groups of the enzyme. Observations on the effect of high oxygen pressure on choline acetylase activity in extracts are consistent with the assumption of the presence of easily oxidizable sulfhydryl groups (194).

On dialysis, choline acetylase rapidly loses its activity (161). Addition of potassium reactivates the system only partly. Further reactivation may be obtained with L-glutamic acid when fresh preparations are used, but not in those obtained from acetone-dried powder (159). Citric acid activates fresh tissue extracts, especially after dialysis, as well as those from acetone-dried powder (159,161). The activating effect of citrate on acetylcholine formation was also observed by Comline in extracts of nonnervous tissue (47). Lipton and Barron suggested that citric acid might be the precursor of active acetate (112). This explanation has, however, been questioned (171). The citric acid effect disappears in more active preparations. In solutions of a still higher degree of purity and of high activity, citric acid has even a marked inhibitory effect, possibly by removing the magnesium necessary for transphosphorylation.

The observation of Nachmansohn, John, and Waelsch (161) that choline acetylase rapidly loses its activity on dialysis and that this activity can be restored only partly by the addition of the compounds mentioned suggested that the enzyme system requires a coenzyme. Such a coenzyme has been found simultaneously and independently by Nachmansohn and Berman (150), Lipmann and Kaplan (110), and Lipton (111). The coenzyme is present in a variety of tissues, like brain, liver, heart, and skeletal muscle,

and appears to be a coenzyme of acetylation generally, rather than that of choline acetylase. If the coenzyme is added, in addition to the other compounds mentioned, a complete reactivation of the dialyzed system may be obtained, even if, in highly active solutions, all activity was lost by dialysis. The coenzyme appears to contain pantothenic acid (110). An activating effect of flavin-adenine-dinucleotide on the enzyme system has been reported by Comline (48). On extracts obtained from human placenta, the effect was nearly as strong as with coenzyme; in brain extracts, however, it was much smaller. For an evaluation of this observation, it would be desirable to test the effect of this compound on highly active preparations.

The choline-acetylating system of rabbit brain has been purified and concentrated by fractional ammonium sulfate precipitation (171a). The procedure used was as follows: Acetone-dried powder or rabbit brain is extracted at 4–6°, with 15 ml. of a solution, per gm. of power, of the following composition: KCl 0.2 M; MgCl<sub>2</sub> 1 mM; Na<sub>2</sub>HPO<sub>4</sub> 6 mM; NaCl 2 mM. The pH is maintained at 7.2. The suspension is centrifuged and the supernatant is fractionated with ammonium sulfate solution. The precipitate formed at 16% ammonium sulfate is inactive and may be discarded. The precipitate obtained when the concentration of ammonium sulfate is increased to 36% contains the bulk of the activity. The precipitate is taken up in about 2 ml. of a solution of the composition described above, supplemented with cysteine 2 mM and dialyzed for 3 hours against the same solution in the cold (4–6°). The enzyme solution keeps at 4° for several days without loss of activity.

The following composition was found to be optimal for the activity of the purified system (in micromoles per ml.): choline chloride 15, sodium acetate 15, adenosinetriphosphate 6, cysteine 30, KCl 40, MgCl<sub>2</sub> 0.7, CaCl<sub>2</sub>, 2, sodium phosphate 1.5; pH 7.0 to 7.2. The added acetate was found to be an essential component. Coenzyme was added in the form of a soluble dry preparation from hog liver at a final concentration of 2 mg. of powder per ml. One milliliter of reaction mixture usually contained 0.15 ml. of enzyme solution. To inactivate any cholinesterase which might still be present, tetraethyl pyrophosphate (10 µg per ml.) was included. The mixture is incubated in test-tubes at 37°. Air may be used in the gas phase, since the amount of cysteine present was found to be sufficient to counteract the inhibitory effect of oxygen.

The activity of the reaction mixture as determined by the bioassay rose from about 0.01 µeq. to 1.5 to 2.7 µeq. (270 to 470 µg) of acetylcholine per ml. at the end of 3 hours. The yield referred to 1 gm. of protein was equivalent to about 180 mg. of acetylcholine chloride per hour. At the concentrations finally used for bioassay (dilution factor of original solution 500 to 1000) the components of the reaction mixture added initially, sepa-

rately or together, failed to elicit contraction of the frog rectus muscle and did not modify the response of the muscle to standard acetylcholine solutions.

### C. ENZYMATIC SYNTHESIS OF A COMPOUND WITH ACETYLCHOLINE-LIKE BIOLOGICAL ACTIVITY

Previous work on the enzymatic acetylation of choline required the use of bioassay. In spite of the physiological importance of acetylcholine no adequate chemical methods were available until recently for the determination of the ester, except by isolation with time-consuming procedures. Recently a simple and rapid colorimetric method for the determination of acetylcholine has been developed by Hestrin (91a). The method is based on the reaction of O-acyl groups with hydroxylamine in alkaline medium and is thus suitable for the determination of the acetyl groups of acetylcholine in the presence of choline and acetate. The amounts of acetylcholine formed in the purified system were found to fall in the range of this method.

When the amounts determined by bioassay were compared with those obtained with the chemical method, a considerable discrepancy was observed between the two methods of analysis (171a). Less than half of the total biological activity of the enzymatically formed product could be accounted for by the chemical method. Acetylcholine added to the reaction mixture at the outset or at the termination of incubation was recovered analytically without loss, both with bioassay and with the chemical procedure. It thus follows that the greater part of the effect obtained in the bioassay is due to a substance which appears to have the same biological action as acetylcholine, but may be distinguished from the latter chemically. In the absence of added choline, the product with biological activity is still synthesized, whereas no acetylcholine is formed when choline is omitted from the reaction mixture. The enzymatically formed product was found to affect the blood pressure of cats and the amplitude of the frog heart in the same way as acetylcholine [Middleton and Middleton (130a)]. Atropine suppressed equally the effect of acetylcholine and of the enzymatically formed product. The experiments show that the acetylating enzyme system derived from brain may form in addition to acetylcholine a second product which exhibits the same biological activity as acetylcholine but differs from this ester chemically.

### D. INHIBITORS

$\alpha$ -Keto acids inhibit the enzyme in fresh brain extracts as well as in those prepared from acetone-dried powder. Particularly striking is the effect of  $\alpha$ -ketoglutaric acid, which may inhibit in  $10^{-4} M$  concentration (158,159).

In the highly active preparation obtained from squid head ganglion, and in extracts prepared from acetone-dried powder of rabbit brain and purified by fractional ammonium sulfate precipitation,  $\alpha$ -keto acids have no effect. The varying effect of citric acid, dependent upon the degree of purity, has been discussed above. The observations show that the activating or inhibitory effects of some intermediate compounds on the activity of an enzyme may depend upon the degree of purity and other accessory conditions of the enzyme preparation. Apparently contradictory observations of this type of effects are often due to the use of differently prepared enzyme solutions. The effects may be the result of the reactions with other inter-

TABLE VI  
ACTIVATORS AND INHIBITORS OF CHOLINE ACETYLASE IN PREPARATIONS  
OF VARIOUS DEGREES OF ACTIVITY<sup>a</sup>

Preparation	ACh formed, mg./g./hr.	L-Glu- tamic acid	Citric acid	$\alpha$ -Keto acids
Fresh brain extract	0.15-0.2	+	+	-
Acetone-dried brain powder	0.6-1.0	0	+	-
Acetone-dried brain powder more ac- tive, complete system	2.0-3.0 (6.0-7.0) <sup>b</sup>	0	0 (-)	-
Acetone-dried brain powder, purified	25.0-30.0 <sup>b</sup>	0	-	0
Squid ganglion	140-150 <sup>b</sup>	0	-	0

<sup>a</sup> + = activation, - = inhibition, 0 = no effect.

<sup>b</sup> Per gram protein.

mediate metabolic processes and not with the enzyme system itself. Table VI illustrates the variations depending upon activity of the preparation used.

In contrast to the effects of intermediate metabolites, changing under various conditions, 2-methyl-1,4-naphthoquinone-8-sulfonic acid inhibits the activity of both crude and purified preparations in about the same concentrations, *i.e.*,  $3 \times 10^{-5} M$ . Fig. 14 shows the effect of this naphthoquinone on extracts obtained from rabbit brain and squid head ganglion. No difference of the action of the drug is observed in these two preparations in spite of the difference of material used and the degree of purity: one forms 6 to 7 mg., the other, 140 to 150 mg. acetylcholine/g. protein/hour. Since the naphthoquinone effect is observed in presence of 0.02 *M* cysteine, it appears more likely that the effect is due to a specific reaction with the

enzyme protein rather than to an oxidoreduction process. Diisopropyl fluorophosphate does not interfere with choline acetylase activity, *in vitro* or *in vivo* (171).

#### E. OCCURRENCE

Choline acetylase was first extracted from brain and electric organ but it is present in all nerve tissue including peripheral fibers free of nerve endings and cell bodies (160). It has been demonstrated in sensory nerves and also in invertebrate tissue (abdominal chain of lobster) (150). It has also been shown to be present in striated muscle (151).

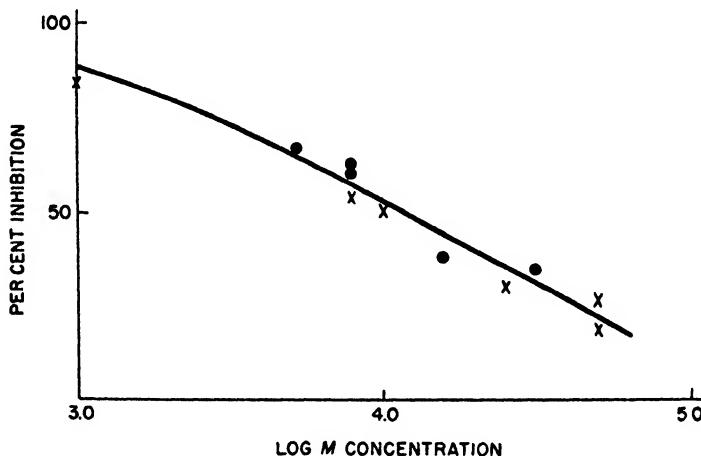


FIG. 14. Inhibitory effect of 2-methyl-1,4-naphthoquinone-8-sulfonic acid (potassium salt) on choline acetylase.

Recently, choline acetylase has been shown to be present in the most primitive phyla containing conductive tissue: in *Neanthes virens*, an annelid, in *Euplanaria maculata*, a flatworm, and in *Tubularia crocea*, a coelenterate (177). The concentrations varied considerably.

Choline acetylase is absent in liver and kidney. A considerable acetylation of choline has been, however, obtained by Comline (47) in extracts of human placenta, up to 0.75 mg./g. acetone-dried powder. The same investigator found a very small activity in extracts prepared from the spleen of several species, about 0.01 to 0.04 mg./g. powder/hour.

A most interesting study was carried out by Stephenson and Rowatt (196) with an acetylcholine-forming enzyme system in bacteria, the *Lactobacillus plantarum* isolated from sauerkraut. Simultaneous fermentation of carbohydrate was necessary. The activity of bacteria grown in absence of pantothenic acid was low. The finer mechanism of acetylation was, however, not elucidated.

The presence of choline acetylase in sensory nerves is of special interest. The absence of acetylcholine in sensory nerves (116,117) was considered for a long time an obstacle for the assumption that this ester is essential for conduction, although Chang *et al.* (42), and, later, Lissák and Pásztor (113) were able to demonstrate the presence of acetylcholine in small amounts. Evidence for the presence of choline acetylase removes definitely the objection to postulating a role of acetylcholine in sensory nerves, since the presence of both choline acetylase and ACh esterase indicates that acetylcholine is metabolized there as well as in motor nerves. This is confirmed by the observations discussed in Section V, A, 3, that inhibition of ACh esterase blocks conduction in sensory as well as in all other types of nerves. It is true that the rate of synthesis in sensory nerves seems to be slower

TABLE VII  
FALL OF CHOLINE ACETYLASE ACTIVITY IN SCIATIC NERVE OF RABBIT

Hours after section	ACh formed, $\mu\text{g}./\text{g}.\cdot\text{hr}.$		Decrease, %
	Normal	Degenerated	
48	87.0	68.4	21.4
48	92.7	68.1	26.7
70	76.0	26.5	65.1
72	73.5	23.0	68.5
72	78.5	26.0	67.5
144	77.0	0	100
150	86.0	0	100

than in motor nerves. In the optic nerve of the rabbit, 15 to 20  $\mu\text{g}.$  acetylcholine/g. nerve/hour is formed as compared to 80 to 110  $\mu\text{g}.$  in a motor nerve (sciatic) of the same animal. These rates are most likely not the maximum, considering the technical difficulties, but quantitative differences of chemical reactions may be expected, and, as was discussed above, the ACh esterase activity in different nerve fibers may also vary considerably.

#### F. CHOLINE ACETYLASE IN DEGENERATING FIBERS

Of considerable physiological interest is the behavior of choline acetylase during the degeneration of the nerve fiber. Evidence that conduction is possible in absence of the enzyme would create a serious difficulty for the assumption of the necessity of acetylcholine for this function. The question has been studied in experiments with the sciatic nerve of rabbit. As may be seen from the figures in Table VII, the enzyme activity falls after

the section of the nerve. However, on the third day, when conduction has disappeared, about one-third of the choline acetylase activity is still present (160). The experiments show that during degeneration, the ability to synthesize acetylcholine in fibers outlasts the ability to conduct impulses.

#### G. DIFFERENCE BETWEEN RATES OF ACETYLCHOLINE FORMATION AND HYDROLYSIS

The difference between the rates of acetylcholine formation and hydrolysis may be briefly discussed. There are two tissues in which these two rates may be compared on the basis of sufficiently established data: the brain and sciatic nerve of the rabbit. In brain extracts, about 300  $\mu\text{g}$ . acetylcholine/g./hour may be formed, whereas 70 to 80 mg. acetylcholine/g./hour may be split. The rate of hydrolysis is in this tissue about 250 times higher than the rate of formation. In the sciatic nerve, about 100  $\mu\text{g}$ . is formed as compared to about 15 to 20 mg./g./hour split. In this case the difference is 150 to 200 times. ACh esterase is an extremely stable enzyme and it appears probable that the maximum activity is measured *in vitro*. Choline acetylase is an extremely unstable and complex system which has to be extracted from tissue. In that case, the actual activity *in vivo* may be higher than that observed *in vitro*. Moreover, ACh esterase is present, as will be seen later, in excess of about ten times. Nothing is known about the excess of choline acetylase, which may be smaller. Thus, the actual difference of rates may be smaller than the figures given indicate.

For an understanding of the problem, the decisive difference which has to be considered is not the difference of rates, but the difference of function. There is a fundamental difference between the function of ACh esterase and that of choline acetylase. If the release of acetylcholine is an essential event in the alterations of the membrane during the passage of the impulse, the active ester has to be destroyed within a millisecond or less, so that the resting conditions may be restored. Therefore, the enzyme which removes the active ester, ACh esterase, must be very active, but only during this brief period, and may then be inactive until the passage of the next impulse. The formation of acetylcholine, on the other hand, need not be such a rapid process. It is generally assumed that the active ester is released from an inactive form. This is supported by the fact that the primary energy released during recovery is used for the synthesis of acetylcholine, thus implying that the synthesis is a slow recovery process. The difference between the two rates therefore does not offer any difficulty and could be expected in the case of two enzymes so different in function and properties.

### V. Effect of Inhibitors of Acetylcholine Esterase on Conduction

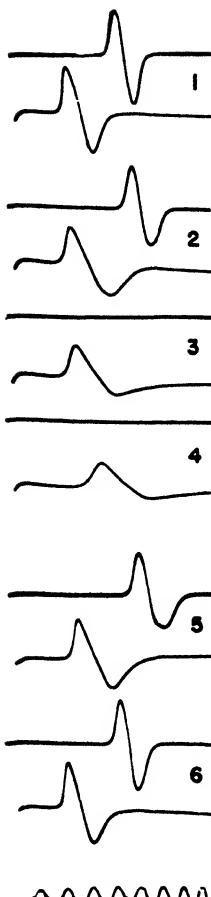
Another and still more direct correlation between ACh esterase activity and nerve conduction has been established by the use of inhibitors of choline ester-splitting enzymes. If the rapid removal of the acetylcholine released during the passage of the impulse is necessary for conduction, interference with the enzymatic mechanism responsible for the removal should block this function.

#### A. REVERSIBLE INHIBITORS

This postulate was first tested in 1945 by Bullock, Nachmansohn, and Rothenberg (37). It has been shown in experiments on the giant axon of squid that, if the nerves are exposed to inhibitors of ACh esterase, like eserine or strychnine, the action potential is altered, and eventually, if the exposure is continued for sufficiently long time, abolished. Since the enzyme-inhibitor complex is easily reversible in the case of these compounds, washing of the nerves should restore conduction. This has been demonstrated with a variety of nerves (35). Fig. 15 shows a typical experiment with the giant axon of squid. After abolition of the action potential by exposure of the nerve to eserine, subsequent washing evokes complete recovery. It is even possible to repeat this cycle several times with the same nerve, as may be seen in Fig. 16, where a frog sciatic nerve was exposed three times to eserine. Each time conduction was abolished completely but after washing, conduction reappeared. After 24 hours, the nerve was in as good a condition as the control nerve (90).

#### B. IRREVERSIBLE INHIBITION OF ACETYLCHOLINE ESTERASE BY DIISOPROPYL FLUOROPHOSPHATE

A new development was initiated by the discovery of diisopropyl fluorophosphate (DFP), a compound which inactivates ACh esterase and other choline-ester-splitting enzymes irreversibly. The discovery of this compound posed two distinct problems: first, whether the high toxicity could be attributed exclusively to the reaction with the choline-ester-splitting enzymes; second, whether it was possible by exposure of nerves to this compound to destroy ACh esterase completely and irreversibly without impairing conduction. As pointed out by Dixon and Needham (64), DFP is one of the most powerful and most specific enzyme inhibitors. Among twenty enzyme systems tested, none was affected except the choline-ester-splitting enzymes. There is growing evidence that compounds which are extremely potent, *i.e.*, act in very low concentrations, produce their effect by interference with enzymes. It appeared then possible to assume that the high toxicity of DFP may be attributed to its action on ACh esterase.

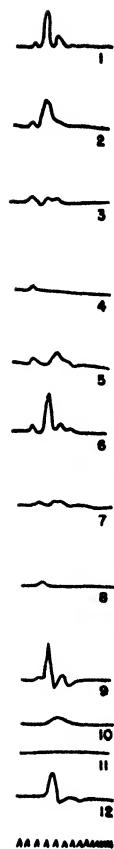


^ ^ ^ ^ ^ ^ ^ ^

FIG. 15

FIG. 15. Effect of  $0.01\text{ M}$  eserine (3.5 mg./ml.) on the action potential of the stellate nerve of squid. Records in pairs, representing potentials from two points on the nerve. (1) action potential of nerve after recovery in sea water, from first exposure to  $0.005\text{ M}$  eserine; (2,3,4) after 1, 3, 11 minutes' exposure to  $0.01\text{ M}$ . The nerve was then put into sea water. (5,6) 8 and 25 minutes' recovery in sea water, 2000 c.p.s.  $25^\circ\text{C}$ .

FIG. 16. Reversible effect on the bullfrog sciatic nerve exposed repeatedly to 6.5 mg. eserine/ml.  $25^\circ\text{C}$ . (1) Normal. First exposure, (2-4) 5, 15, and 21 minutes; eserine replaced by Ringer solution; (5-6) 15 and 48 minutes recovery. Second similar exposure, (7,8) 9 and 17 minutes; (9) retested 12 hours later after having been kept in Ringer solution. Third exposure, (10,11) 5 and 73 min.; (12) 134 minutes recovery. 1000 c.p.s.



^ ^ ^ ^ ^ ^ ^ ^

FIG. 16

Early observations with this compound seemed to contradict the assumption of the necessity of ACh esterase for conduction. Several difficulties

and apparent contradictions had to be overcome before a clear picture was obtained.

### 1. Reversibility of Acetylcholine esterase Inhibition by Diisopropyl Fluorophosphate

The irreversible inactivation of ACh esterase by DFP was believed to be instantaneous. Since abolition of conduction in nerves exposed to DFP can be reversed, a completely irreversible immediate destruction of ACh esterase appeared incompatible with the assumption that the effect on con-

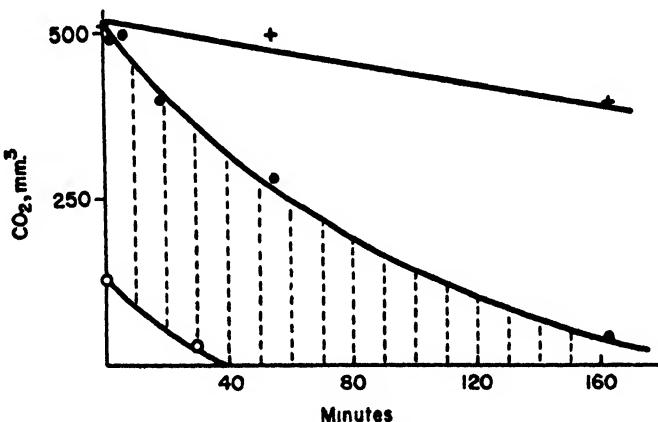


FIG. 17. Reversibility of ACh esterase inhibition by DFP *in vitro*, tested by dilution method. Electric tissue esterase, 9°C. Without DFP the solution liberated 790 mm.<sup>3</sup> carbon dioxide/hour. +—+ Activity in presence of 0.1 µg. DFP/ml. ○—○ Activity in presence of 0.5 µg. DFP/ml. ●—● Activity after incubation with 0.5 µg. DFP/ml. for varying periods of time and subsequent dilution to 0.1 µg./ml. Dotted lines indicate reversibility as a function of time.

duction is due to the inactivation of ACh esterase. It was suggested that the conduction in nerves exposed to DFP is blocked by a general toxic effect of this compound. It could be shown, however, that the irreversible inactivation of ACh esterase by DFP is not an instantaneous process but progresses rather slowly. The rate of irreversible inactivation depends upon a number of controllable factors, like temperature, concentration of inhibitor, and others (167). It could be demonstrated by the dilution method that at low temperature (9°C.) the process may be partly reversed even after 2 to 3 hours' incubation of the enzyme with DFP (Fig. 17). The higher the temperature, the higher is the rate of the irreversible process, the  $Q_{10}$  being about 2. At 37°C., nearly all of the enzyme is irreversibly inactivated in about 35 minutes (Fig. 18). The concentration of the inhibi-

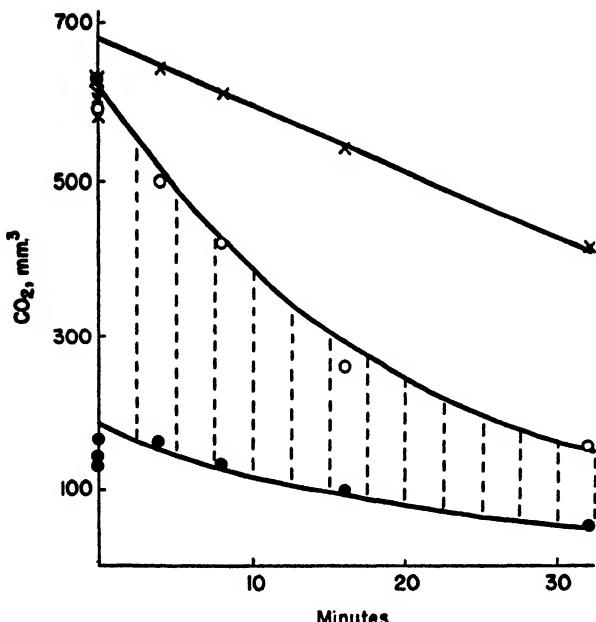


FIG. 18. Reversibility of ACh esterase by DFP at 37°C. Esterase from nucleus caudatus of ox brain. After incubation with 0.8 µg. DFP/ml., the inhibitor was diluted to 0.1 µg./ml. Symbols as in Fig. 17.

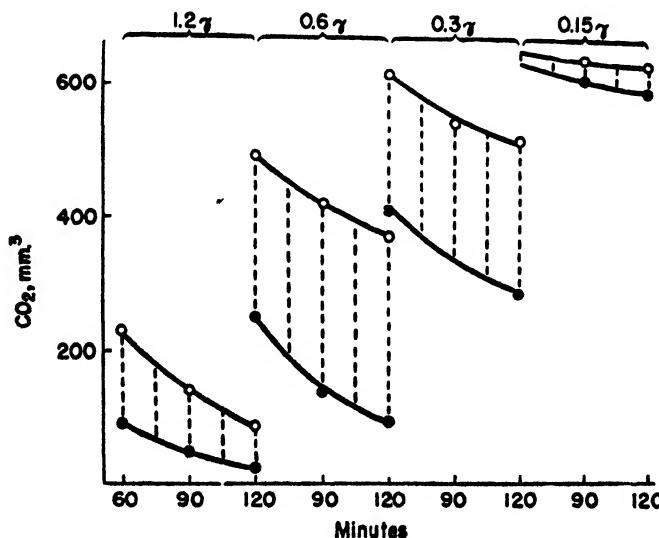


FIG. 19. Reversibility of ACh esterase inhibition after incubation with concentrations of DFP for 60–120 minutes at 7°C., tested by dilution method. ACh esterase from electric tissue. Symbols as in Fig. 17.

tor is an important factor. For a given time of incubation, the range of the concentration at which reversibility may be demonstrated is rather small. This is illustrated in Fig. 19.

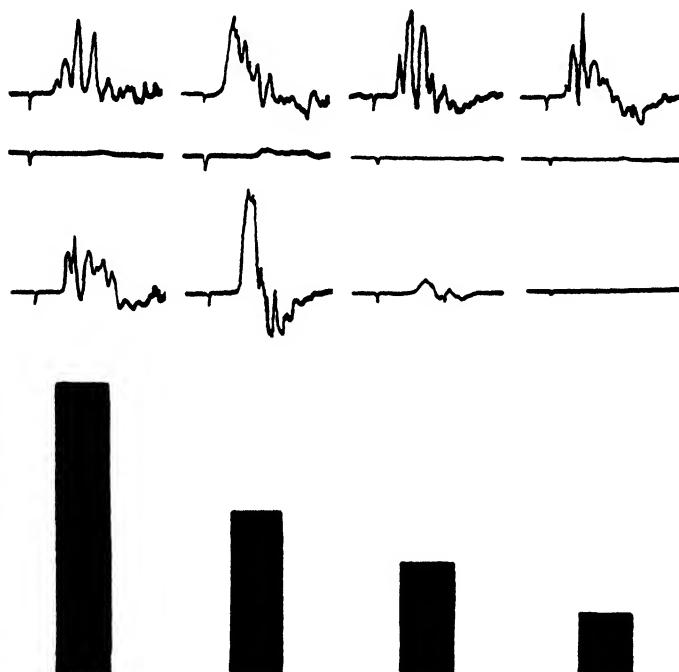


FIG. 20. Parallelism between progressive irreversible abolition of conduction and progressive irreversible inactivation of ACh esterase in lobster nerves exposed to DFP. Four nerve preparations shown in the four columns were kept in DFP (0.013 M) for 30, 60, 90, and 120 minutes, respectively and then washed in sea water. Top line of each column shows action potential in untreated nerves. Second line shows abolition of response by DFP. Third line shows degree of recovery of action potential after prolonged washing of nerve. ACh esterase activity still present is shown in fourth line. Carbon dioxide output is 233, 129, 88.5 and 50 mm.<sup>3</sup>/100 mg./hour, as compared to about 2000 mm.<sup>3</sup> in the normal preparation.

## 2. Parallelism between Abolition of Conduction and Inactivation of Acetylcholine esterase

The peculiar feature of ACh esterase inhibition by DFP, *viz.*, its slowly progressive irreversibility, made it possible to establish conclusively the necessity of ACh esterase for conduction. A most striking parallelism has been demonstrated between the progressive irreversible inactivation of ACh esterase activity and the progressive irreversible abolition of conduction. The giant fibers which run through the abdominal chain of the

lobster were selected for these experiments, since the ACh esterase activity here is high and the preparation therefore favorable for correlating electrical and chemical processes (36). The nerves were exposed to DFP for varying

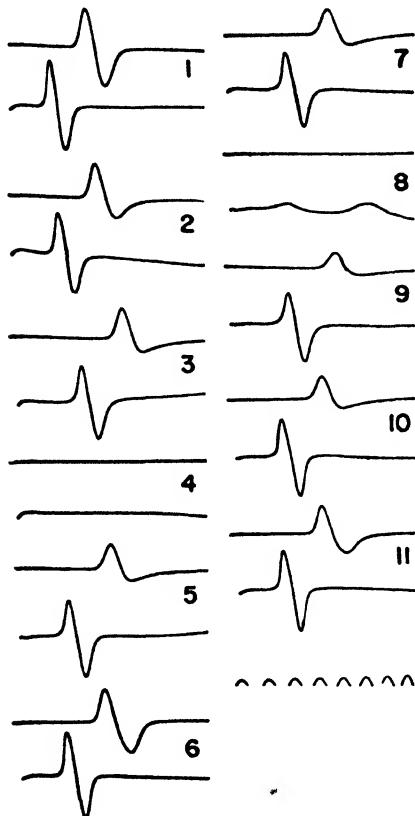


FIG. 21

Fig. 21. Effect of 0.005 M DFP (1 mg./ml.) on action potential of stellar nerve of squid. Records in pairs, representing potentials from two points on the nerve. (1) before exposure; (2,3,4) after 6, 7, 5, and 12 minutes' exposure, respectively. The nerve was then washed with sea water; (5,6) after 4, 5, and 105 minutes' recovery. Second exposure to DFP. (7,8) after 5 and 8 minutes' exposure. (9,10,11) effect of 2, 7, and 66 minutes' recovery in sea water. 2000 c.p.s. 23°C.

Fig. 22. Reversible effect on the bullfrog sciatic nerve, part of which was exposed to DFP (4 mg./ml.) for 85 minutes. Left, recordings of unexposed part. (1) normal; (2,3) exposure 11 and 25 minutes; (4-6) recovery 25, 75, and 135 minutes. Room temperature. 1000 c.p.s.

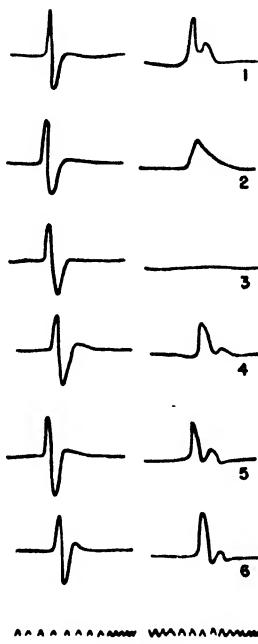


FIG. 22

periods of time and then washed with sea water. The longer the period of exposure and the less complete the recovery of the action potential, the smaller was the activity of ACh esterase remaining in the nerves (Fig. 20).

When conduction was irreversibly abolished, the activity of the enzyme was 3% or less of the initial. The same parallelism has been obtained in experiments with other nerves, especially also with the giant axon of squid, a single-fiber preparation. In all instances in which conduction could be restored, ACh esterase activity was still present (35). Figs. 21 and 22 illustrate the reversible abolition of conduction of the giant axon of squid and of the frog sciatic exposed to DFP. Fig. 23 shows an experiment in

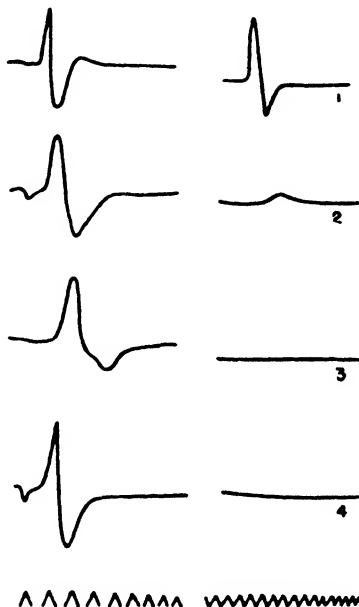


FIG. 23. Irreversible effect on bullfrog sciatic nerve exposed to DFP, as in Fig. 22 but for 120 min. (1) normal; (2) 25-minute exposure. After 120 minutes' exposure returned to Ringer solution. (3,4) 45 minutes and 5 hours later. Room temperature. 1000 c.p.s.

which the abolition became irreversible after exposure of the nerve (frog sciatic) for 120 minutes to DFP.

The parallelism between ACh esterase activity and conduction has been established not only as a function of time, but also as a function of temperature. At room temperature it takes about 100 minutes to abolish conduction irreversibly. At 5 to 10°C. about 4 hours is required for the same effect, as could be expected on the basis of the rate of the irreversible inactivation of ACh esterase *in vitro*. According to these data, it should take about 30 minutes to produce the same effect at 37°C. This has been tested with the superior cervical sympathetic nerve of the cat (90). If the nerves are exposed for a few minutes and the action potential abolished,

washing with Ringer solution restores conduction (Fig. 24), but in about 30 minutes the process becomes irreversible (Fig. 25). Here again, ACh esterase is present as long as the action potential reappears. Little ACh esterase is found or none if conduction is irreversibly abolished after exposure to DFP. If the same nerve preparation is exposed to eserine, conduction comes back completely even after 50 minutes' exposure (Fig. 26).

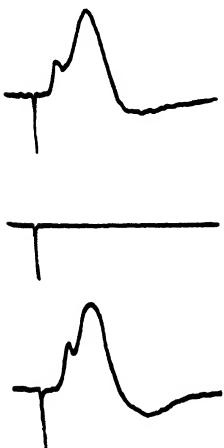


FIG. 24

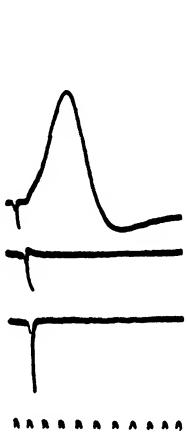


FIG. 25

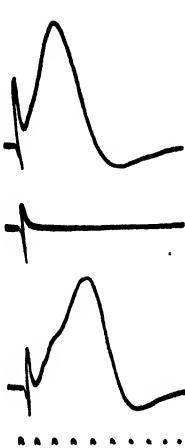


FIG. 26

FIG. 24. Reversible effect on superior cervical sympathetic nerve of the cat exposed to DFP for 2 minutes. Top to bottom: normal; after 2-minute exposure; recovery. 1000 c.p.s.

FIG. 25. Irreversible effect on superior cervical sympathetic nerve of the cat exposed to DFP for additional 20 minutes after abolition of the action potential. Top to bottom: normal; after 3-minute exposure; after 120 minutes in Ringer solution. 1000 c.p.s.

FIG. 26. Reversible effect on superior cervical sympathetic nerve of the cat exposed to eserine (6.5 mg./ml.) for additional 20 minutes after abolition of the action potential. 37°C. Top to bottom: normal; after 8-minute exposure; 67-minute recovery. 1000 c.p.s.

### 3. Generality of the Role of Acetylcholine in Conduction

The blocking of conduction by inhibitors of ACh esterase could be demonstrated with all types of nerves, myelinated and unmyelinated, vertebrate and invertebrate, motor and sensory nerves, adrenergic as well as cholinergic nerves. A few examples may be given. In Figs. 27 and 28, two experiments are shown with purely sensory nerves, the optic and superficial ophthalmic of *Raja erinacea*. The nerves were exposed to DFP and the abolition of conduction was observed within a few minutes. Immediate

washing of the nerves in sea water led, as in all other nerves, to complete recovery. As a sample of a so-called adrenergic nerve, the splanchnic nerve of the bullfrog was selected. This nerve is composed of slowly conducting C-fibers together with a very few larger fibers. Although a certain proportion of preganglionic fibers cannot be excluded, the nerve undoubtedly contains a large number of postganglionic sympathetic fibers, which

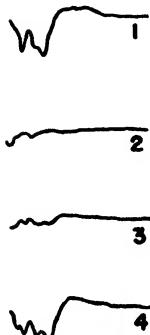


FIG. 27

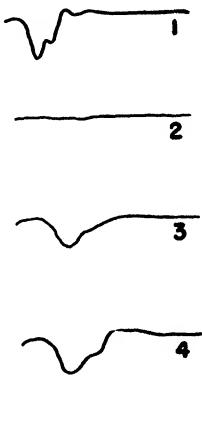


FIG. 28

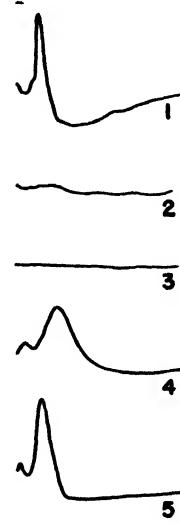


FIG. 29

FIG. 27. Effect of  $0.015\text{ M}$  DFP on action potential of optic nerve of *Raja*. (1) before exposure to DFP; (2) after 4-minute exposure; (3) first reappearance after 5 minutes in sea water, following complete abolition by DFP; (4) after 16 minutes in sea water. 1000 c.p.s.  $24^\circ\text{C}$ .

FIG. 28. Effect of  $0.02\text{ M}$  DFP on action potential of superficial ophthalmic nerve of *Raja*. (1) before exposure to DFP; (2) after 6-minute exposure; the nerve was then put back into sea water; (3,4) after 1 and 2 hours' recovery. 2000 c.p.s.  $25^\circ\text{C}$ .

FIG. 29. Effect of  $0.02\text{ M}$  eserine on splanchnic nerve of bullfrog. (1) action potential before exposure, (2,3) after 3 and 6 minutes in eserine; the nerve was then put back into frog Ringer solution; (4,5) 4 and 5 minutes later. 66 c.p.s.  $23.5^\circ\text{C}$

are classically considered to be adrenergic. As may be seen in Fig. 29, on exposure to eserine the action potential was rapidly impaired. After 6 minutes, it was completely abolished, indicating that all elements including the adrenergic fibers were affected. The nerve was then put back into fresh Ringer solution. After 5 minutes, recovery was visible and, after 20 minutes, nearly complete (34).

Since the electrical manifestations of conduction in nerve and muscle are

in many respects similar, it appeared possible to assume that the mechanism of conduction is the same in both tissues. As discussed previously, ACh

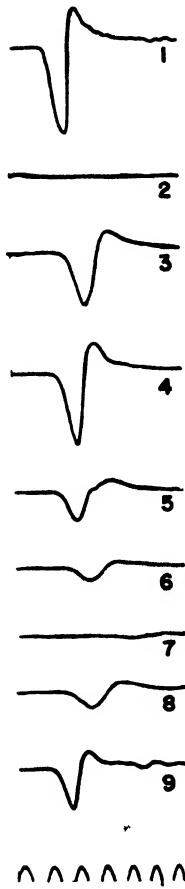


Fig. 30

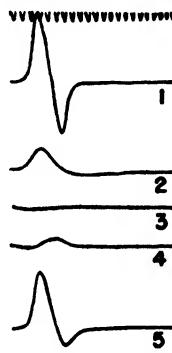


Fig. 31

FIG. 30. Effect of 0.01 M DFP on muscle action potential of curarized frog sartorius muscle. (1) action potential before exposure; (2) 9 minutes later, after which muscle was returned to Ringer-curarine; (3,4) 3 and 10 minutes later. Then second exposure to DFP. (5-7) 2, 5, and 10 minutes in DFP solution. Again back in Ringer solution. (8,9) 5 and 15 minutes' recovery. 200 c.p.s. 24°C.

FIG. 31. Effect of 0.01 M eserine on the action potential of curarized frog sartorius muscle. (1) action potential before exposure to eserine; (2,3) after 3 and 9 minutes' exposure. Muscle put back into Ringer-curarine solution; (4,5) after 7 and 70 minutes' recovery. 1000 c.p.s. 23.5°C.

esterase was found to be present in all muscle tissue throughout the whole animal kingdom. Presence of choline acetylase was also demonstrated in muscle. As illustrated in Figs. 30 and 31, exposure of muscles to DFP or

eserine abolishes the action potential reversibly. In order to ensure direct stimulation of the muscles and to exclude any effect on the end plate, the compounds were used only after complete curarization (34,52).

The ubiquity of ACh esterase in all conductive tissue, nerves, and muscle, and the dependence of conduction upon the activity of this enzyme, thus permits the conclusion that the role of acetylcholine in conduction of impulses is a general one. As was pointed out by Bremer (30), if the acetylcholine - ACh esterase mechanism were limited to one type of nerve only, this fact would not be an insurmountable obstacle for attributing an essential role to the system in conduction. The evidence for the generality of this mechanism, obtained in the meantime, certainly contributes to making the picture more satisfactory.

#### *4. Impossibility of Dissociating Acetylcholine esterase Activity and Conduction*

It is impossible under any condition to dissociate conduction and ACh esterase activity. It is true that it is sometimes difficult to demonstrate ACh esterase activity in nerves which were exposed to DFP. This difficulty, however, is due to the fact that normally the enzyme is present in excess, which apparently amounts in some nerves as e.g., the frog sciatic nerve, to about ten to twelve times the concentration necessary for normal function. In other words, 90 to 92% of the ACh esterase present in this nerve may be inactivated without impairing conduction, but the remaining 8 to 10% is essential. An excess of ten times is not unusual, on the basis of our experience with other enzyme systems. An excess of 50 to 100 times may be found. But, whereas the normal ACh esterase activity in most nerves is easily determinable, the remaining activity after inactivation of the excess is sometimes small in absolute figures and its determination therefore requires special precaution. In the experiments of Crescitelli, Koelle, and Gilman (57) on the bullfrog sciatic nerve, the manometric technique used was inadequate for detecting the remaining ACh esterase activity, due to several adverse factors. An activity equivalent to 7 to 10% of the initial activity can, however, be demonstrated in the following way. The homogenized nerves are added to a solution of acetylcholine and the disappearance of the ester is measured by the determination of the acetylcholine with the frog rectus method before and after incubation (35).

Boyarski, Tobias, and Gerard tried in another way to dissociate ACh esterase and conduction (27). They exposed a frog sciatic nerve to DFP for a threshold time and in a threshold concentration which did not yet affect conduction. Under these conditions, they were unable to find any ACh esterase activity, using for the determination of acetylcholine, before and after incubation, the frog rectus method. Unfortunately, in their experiments the ratio of enzyme to substrate was inadequate, since too

small amounts of tissue were used. Exposing the frog sciatic to the same threshold concentration and for the same threshold time, leaving conduction still unimpaired, it was found that the nerve contains ACh esterase concentration capable of hydrolyzing 400 to 500  $\mu\text{g}$ . acetylcholine/g./hour if an adequate ratio of enzyme to substrate is used (75). These are minimum values because the substrate concentration may have been below the optimum for the enzyme activity, since it had to be kept low for the particular method used. Since exposure for a longer period of time or to higher concentrations impaired conduction, these experiments are not only a striking demonstration of the necessity for ACh esterase but at the same time give an approximate indication of the minimum enzyme activity required and the excess present.

For the evaluation of the enzyme activity required for maintaining conduction, it may be useful to recall the small initial heat in nerves. The initial heat/g./impulse in a frog sciatic nerve at 23°C., at a rate of 300 to 400 impulses/second, is about  $2$  to  $3 \times 10^{-8}$  gcal. If acetylcholine acts as a trigger in the chain of reactions associated with the flow of current, the hydrolysis of the ester will account for only a part of the initial heat. Assuming that the hydrolysis accounts for 25% of the initial heat or 5 to  $7 \times 10^{-9}$  gcal., this would amount to 0.0003 to 0.0004  $\mu\text{g}$  of acetylcholine hydrolyzed per g. impulse calculated on the basis of 3100 g. cal. released/mole acetylcholine split. The ability to split 400 to 500  $\mu\text{g}$  of acetylcholine/g. nerve/hour would then be adequate to account for one to two million impulses conducted during this period.

##### *5. Coincidence of Acetylcholine esterase Inactivation with Death*

If ACh esterase activity is essential for conduction, survival of an animal should be impossible in the absence of ACh esterase. Observations were reported, however, that animals may survive in complete absence of the enzyme in brain (125). The question has been re-examined. It was found, with experiments on rabbits, that absence of ACh esterase in brain coincides always with death. If 0.3 mg. DFP/kg. is injected into rabbits, some animals survive, others die. In the surviving animals, ACh esterase was always present in the brain, without a single exception. In the others, the enzyme had disappeared. In a large series of experiments, the acetylcholine split in brain cortex of normal rabbits was found to be on the average about 45 mg./g./hour, in the nucleus caudatus, about 316 mg./g./hour. Following the injection of DFP, the average activity found in the cortex of the surviving animals was about 12 mg. acetylcholine split/g./hour or 26% of the initial activity, in the nucleus caudatus it was about 70 mg., or 22%. The lowest values observed were about 10% of the normal average, a few were close to 50% (157). During recovery from DFP poisoning, the signs of toxicity diminish as the brain ACh esterase rises (79).

Studies on *Ambystoma* embryos have confirmed the observations that acetylcholine esterase is present in considerable excess of that needed for minimum activity (26a). In embryos reared in DFP solution acetylcholine esterase increases, although the absolute level of enzyme activity is much lower in treated than in untreated animals of the same morphological stage, being on the average only 10 per cent of the control values. This figure is in good agreement with the minimum value found to be necessary for unimpaired conduction.

In striking contrast to the complete inactivation of ACh esterase activity in the animals which died following the injection of DFP, respiration, glycolysis, succinic dehydrogenase, ATPase and other enzymes tested were not affected.

#### 6. Concentration of Diisopropyl Fluorophosphate Required for Abolition of Conduction

One of the main objections to the assumption that DFP abolishes conduction by inactivation of ACh esterase is the concentration used. DFP inactivates ACh esterase *in vitro* in a concentration of a few  $\mu\text{g./ml.}$ , whereas the concentrations used to abolish conduction are 1 to 2 mg. DFP/ml. This difference has been considered as evidence that the abolition of conduction by DFP is due to a general toxic effect, rather than to the inactivation of ACh esterase. This objection does not take into account one of the most fundamental factors on which the action of a compound applied to a living cell depends, *viz.*, the structure. The concentration outside the cell is irrelevant. The decisive factor is the concentration at the site of action. All nerve fibers are surrounded by a myelin sheath and by other membranes which may form either a complete barrier for the penetration of some compounds into the interior or slow down the rate of penetration. The concentration of DFP in the interior of the cell has been determined at the time when the action potential disappears, in experiments with the giant axon of squid. As the figures in Table VIII show, the concentration is of the order of magnitude of 1  $\mu\text{g./g.}$ . Sometimes it is lower and in one case it has been about 5  $\mu\text{g./g.}$  (75).

#### C. KINETICS OF ACETYLCHOLINE ESTERASE INHIBITION

The question of the necessity of ACh esterase for conduction has been unequivocally answered. Whereas at first the observations on the action of DFP appeared to be a challenge to the postulate of an essential role of acetylcholine in conduction, this idea emerged from the later investigations stronger than before. The compound has been an extremely valuable tool for elucidating several aspects of the special physiological problem. In addition, studies with this compound have provided valuable information concerning the question of general toxicology, *viz.*, whether the action upon

the enzyme is responsible for the variety of effects produced. All indications described so far favor the assumption that the toxic effects must be attributed to this single chemical reaction. Further support is the interesting observation of Krop that of certain compounds structurally related to DFP only those inactivating the ACh esterase rendered the muscle inexcitable (103a,b). Significant in this respect also is the recent finding of Jones, Meyer and Karel (99). The acute toxicity of a great number of organic phosphorus compounds tested by these investigators was found to be a function of their potency as ACh esterase inhibitors. The reaction between enzyme and inhibitor depends, however, upon a great number of factors. Many of them may be demonstrated *in vitro*, still more will determine the effect *in vivo*.

The effects of temperature and inhibitor concentration on the reversibility of ACh esterase inhibition were already mentioned. The study of

TABLE VIII  
CONCENTRATION OF DFP IN AXOPLASM OF GIANT AXON OF SQUID  
EXTRUDED WHEN ACTION POTENTIAL HAD BEEN ABOLISHED

DFP conc. outside, $M \times 10^{-3}$	Time of expos- ure, min.	Concentration of DFP inside		
		$M \times 10^{-6}$	$\mu\text{g./g.}$	Per cent of outside
2.2	44	2.0	0.36	0.09
2.2	38	8.4	1.5	0.38
5.5	11.5	30.0	5.4	0.54
5.5	4	2.5	0.45	0.05
5.5	27	1.3	0.23	0.02

the kinetics of the inhibitory action is necessary for the understanding of its complexity. The inhibition of ACh esterase by DFP occurs on a mole-to-mole basis (167). In these experiments, an enzyme solution has been used which contains only one component in the analytical run in the ultracentrifuge so that it was possible to estimate the enzyme concentration. The number of molecules destroyed irreversibly by incubation with DFP increases in direct proportion to the number of inhibitor molecules (Fig. 32). In this connection it may be recalled that Goldstein (85) in an extremely relevant study of the kinetics of serum esterase has offered evidence that eserine acts on that choline-ester-splitting enzyme also on a mole-to-mole

A second factor on which the inhibitory effect depends is the concentration of the enzyme in relation to the inhibitor concentration. An excess of inhibitor is required which rises with decreasing enzyme concentration.

The excess of inhibitor necessary to produce 50% irreversible inhibition in a given time has been tested. If  $pE$ , the negative logarithm of the molar concentration of the enzyme, is plotted against the logarithm of the ratio of inhibitor to enzyme concentration, a straight line is obtained (Fig. 33). In a solution in which 1 ml. splits 12 g. acetylcholine/hour, the excess of molecules of inhibitor over molecules of enzyme is 25. In a solution in which 1 ml. splits 1 mg. acetylcholine/hour, which is a concentration of the order of magnitude used generally with the Warburg manometric method, more than 100,000 molecules of inhibitor are necessary for each molecule of enzyme in order to obtain the 50% inhibition (171).

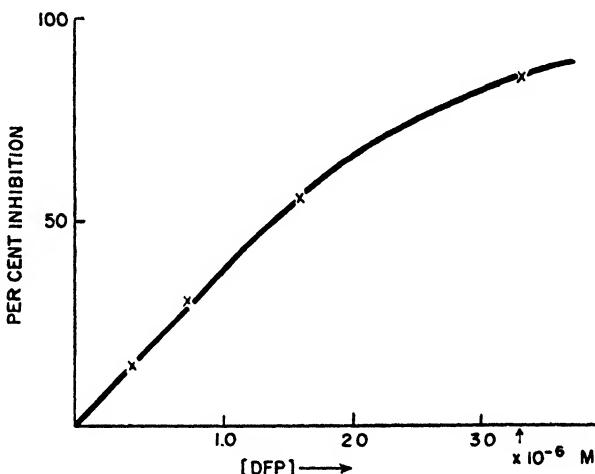


FIG. 32. Relationship between enzyme inhibition, in per cent, and inhibitor concentration. Amount of enzyme inactivated irreversibly by incubation with DFP increases at least up to 50% in direct proportion to DFP concentration.

The importance of substrate concentration for enzyme activity has been known since the classical observations of Michaelis and Menten (130) and a discussion of the theories concerning this question may be found in Haldane's book (91). The effects of substrate concentration on the action of inhibitors have been analyzed by Lineweaver and Burk (107), who discussed different theoretical aspects of the kinetics of such effects. The role of the substrate concentration for the effect of some inhibitors of choline-ester-splitting enzymes has been investigated mainly by Straus and Goldstein (197), Goldstein (85), and Augustinsson (12).

The experiments in which ACh esterase activity has been tested as a function of substrate concentration have been described. The situation becomes more complex in the presence of inhibitors since the activity of

such a system will depend upon the concentration of all three components, enzyme, substrate, and inhibitor. The effect of substrate concentration upon the inhibition by DFP in different concentrations on the highly purified preparation obtained from electric tissue of ACh esterase has been recently investigated by Augustinsson and Nachmansohn (14). Fig. 34 illustrates this effect. The optimum of the activity of pS curves does not change in the presence of DFP. Similar activity - substrate concentration relationships have been obtained with tetraethyl pyrophosphate (TEPP). This substance, like DFP, belongs to a series of organic phosphorus-contain-

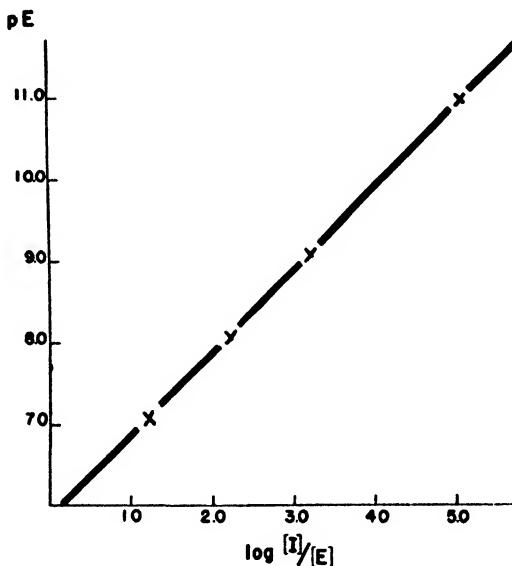


FIG. 33. Excess of DFP required for varying enzyme concentrations. pE, the negative logarithm of the molar concentration of enzyme is plotted against the logarithm of the ratio of inhibitor concentration [I] to the enzyme concentration [E].

ing compounds which are extremely potent inhibitors of ACh esterase. TEPP, like DFP, inactivates this enzyme irreversibly but it acts in much lower concentrations and the irreversible inactivation occurs much more rapidly. After 2 minutes' incubation, Augustinsson and Nachmansohn were unable to demonstrate any reactivation by the dilution technique with which, as described before, under similar conditions, the inhibition of ACh esterase by DFP could be partly reversed for a considerable period of time, *i.e.*, for at least 2 hours or more.

As to the mode of the action of inhibitors like DFP and TEPP, as compared to that of the alkaloids prostigmine and eserine, attention has been

mostly focused upon the irreversible effect of the former group in contrast to the reversible action of the latter. There are other remarkable differences between the four compounds revealed by the study of the kinetics of the inhibition. The sequence in which the three components, enzyme, inhibitor, and substrate, are combined affects the course of hydrolysis in the case of the competitive and reversible inhibition by prostigmine and eserine in a manner different from that found in the cases of DFP and

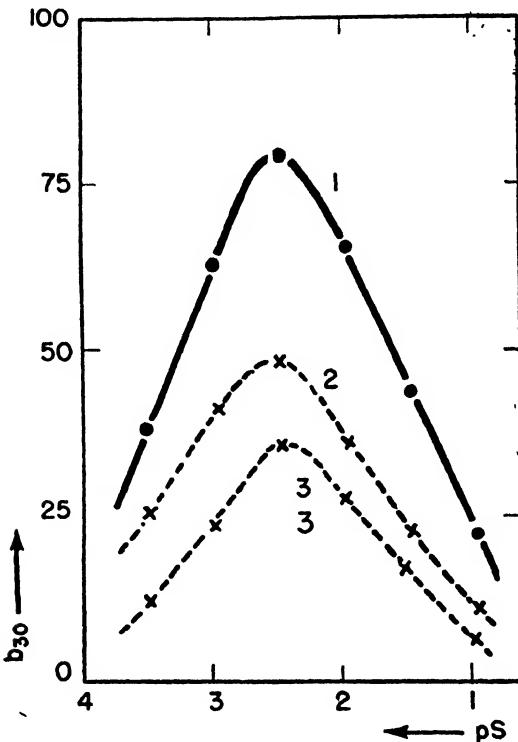


FIG. 34 Activity-pS curves for purified ACh esterase (from electric tissue of *Electrophorus electricus*) in presence of DFP (14). Abscissas and ordinates as in Fig. 4. (1) control, (2,3) DFP in 1 and  $2 \times 10^{-6} M$  concentration.

TEPP. With the alkaloids, the degree of inhibition is the same after equilibrium has been reached, whether the enzyme is first incubated with the inhibitor or whether substrate and inhibitor are added simultaneously. Before the attainment of an equilibrium, a period which lasts 10 to 25 minutes, depending upon the concentrations used, the inhibition is stronger than during equilibrium when the enzyme has been incubated with the inhibitor. Without incubation, the inhibition is weaker during the period

before equilibrium. In the case of irreversible inhibition by DFP and TEPP, there is no equilibrium attained. However, even in this case, if acetylcholine is added before the inhibitor, much higher concentrations of the latter become necessary to obtain the same degree of inhibition as that observed after incubation of the enzyme with inhibitor. The protective effect of acetylcholine is particularly conspicuous in the case of DFP. The effect suggests that these compounds act on the same active center of the

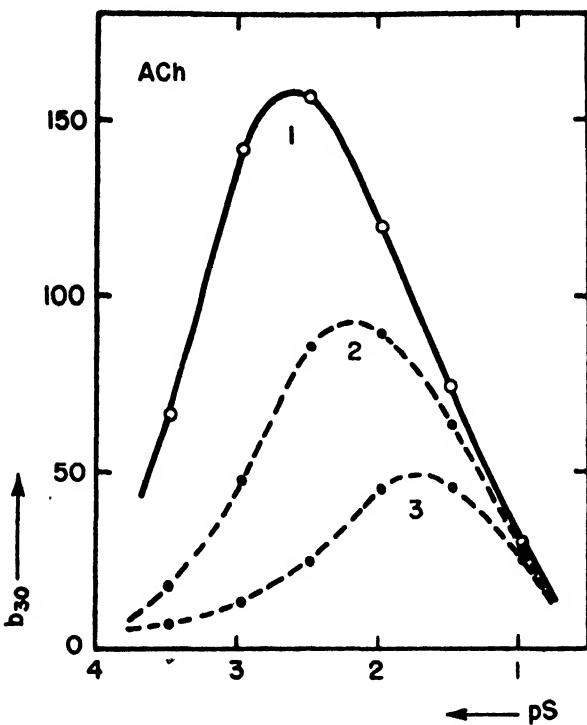


FIG. 35. Activity-pS curves for ACh esterase of cow erythrocytes in the presence of added choline (12). Abscissas and ordinates as in Fig. 4. (1) control, (2,3) choline in  $0.9 \times 10^{-2}$  and in  $3.6 \times 10^{-2} M$  concentration.

enzyme molecule as does acetylcholine. The assumption is supported by the observation that prostigmine in the same concentration as DFP has a strong protective action. The protective effect is obtained with very low concentrations of prostigmine if compared to those of acetylcholine. The protection of the enzyme with prostigmine against DFP may be complete if both inhibitors are used in equimolecular concentration. Eserine protects the enzyme markedly less against DFP than does prostigmine. None of the alkaloids protect the enzyme against TEPP if equimolecular concen-

trations are used. This may be explained by a much higher affinity of TEPP for the enzyme since TEPP inhibits the enzymatic activity in so much lower concentrations.

The data available are in favor of the assumption that both types of inhibitors may act on the same center which forms with one type a reversible and with the other an irreversible complex. Although we do not know the underlying chemical reactions in this case, similar situations are

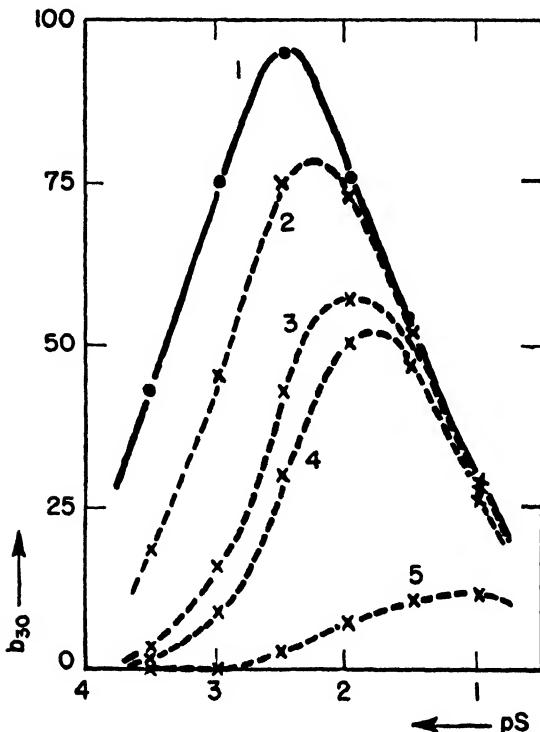


FIG. 36. Activity-pS curves for purified ACh esterase (from electric tissue of *Electrophorus electricus*) in presence of prostigmine (14). (1) control, (2-5) prostigmine in  $0.4$ ,  $1$ ,  $2$ , and  $10 \times 10^{-4} M$  concentration. Abscissas and ordinates as in Fig. 4.

well known. For example, the combination of hemoglobin with oxygen may be inhibited by carbon monoxide or potassium ferricyanide. Both inhibitors combine with the iron of the prosthetic group, the iron porphyrin. The carbon monoxide effect is easily reversible whereas the potassium ferri-cyanide reacts with the iron irreversibly.

The effect of substrate concentration shows another interesting difference between the alkaloids and the organic phosphorus-containing inhibitors.

In the presence of prostigmine, the optimum of substrate concentration shifts to high concentrations as is typical for competitive inhibition. Such a type of ACh esterase inhibition was shown by Augustinsson (12) with choline (Fig. 35). The same type of curve has been obtained with prostigmine, as illustrated in Fig. 36, but in much lower concentrations since prostigmine has a much greater affinity. The results obtained with eserine resemble those with prostigmine. However, the shift of optimum is much less pronounced (14). In contrast, with DFP and TEPP, there is, as mentioned above, no shift at all of the optimum substrate concentration.

The experiments stress the impossibility of considering the inhibitory effect independent of the substrate concentration. Prostigmine inhibits very strongly at low concentrations of acetylcholine but the effect decreases with increasing substrate concentration and becomes close to zero at high substrate concentration. Thus, the effect may appear smaller with high inhibitor concentrations than with low ones if different substrate concentrations are used. Furthermore, in low acetylcholine concentrations, both prostigmine and eserine if applied at the same concentration inhibit the enzyme to about the same degree. At high substrate concentrations, where prostigmine does not affect the enzyme activity at all, eserine may have a strong effect. The observations show how difficult the interpretation may become under physiological conditions. When the compound is applied to the cell or tissue or to the whole animal, the effect may be still less predictable in view of the many additional factors, like circulation, permeability, rate of penetration, and others. It is thus not difficult to see that, even if the toxic signs of DFP must be attributed to the reaction with ACh esterase, the picture may be influenced by a great variety of accessory factors. They are apparently responsible for the complexity of the effects produced, but this does not justify the assumption of a general toxic effect.

## VI. Difference between Axon and Synapse

### A. IMPERMEABILITY OF AXON SURFACE MEMBRANES TO METHYLATED QUATERNARY AMMONIUM SALTS

The investigations on the effects of ACh esterase inhibitors led to an elucidation of some of the problems connected more specifically with synaptic transmission of the nerve impulse in contrast to axon conduction. The studies of the events at the synaptic junctions were responsible for the hypothesis of neurohumoral or chemical transmission and it appeared desirable to find a satisfactory explanation for these findings and to integrate them with the facts established concerning axon conduction.

In contrast to the inhibitors of ACh esterase discussed, prostigmine has no effect on conduction in nerve and muscle. Fig. 37 shows the action potential of the giant axon of squid before and after exposure to prostigmine

in  $10^{-2} M$  concentration. In Fig. 38 is shown the same experiment carried out with the fin nerve of squid, a multifiber preparation, and continued for as long as 370 minutes (37). In spite of the high concentration used and the long time of exposure, conduction was unaffected. The same is true for the muscle fiber. Fig. 39 shows the action potential of a curarized frog sartorius exposed to  $10^{-2} M$  prostigmine. Here again, in contrast to



FIG. 37

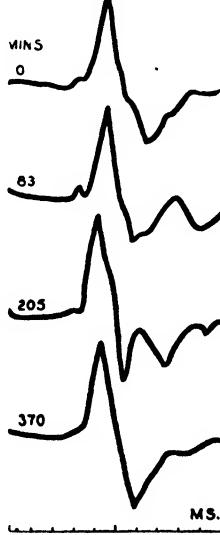


FIG. 38

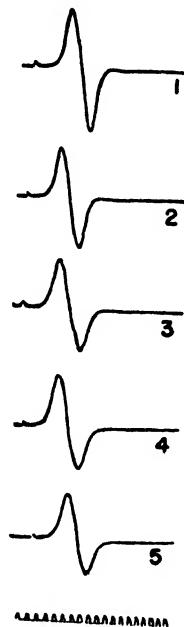


FIG. 39

Fig. 37. Effect of  $0.01 M$  prostigmine on single-fiber action potential (giant axon). Records before and after 45 minutes.

Fig. 38. Effect of  $0.01 M$  prostigmine on fin nerve. Records before and after 83, 205, and 370 minutes. Traced from enlarged photographs.

Fig. 39. Effect of  $0.01 M$  prostigmine on action potential of curarized frog sartorius muscle. (1) action potential before exposure to prostigmine. (2-4) after 10, 40, and 60 minutes' exposure. Muscle put back into Ringer-curarine solution. (5) after 20-minutes recovery. 1000 c.p.s.  $24^{\circ}\text{C}$ .

the action of eserine and DFP, conduction remained virtually unchanged (34).

Prostigmine is, *in vitro*, a very strong inhibitor of ACh esterase, at least as strong as eserine. There is, however, an important difference as to the chemical constitution and properties of the two compounds. Eserine is a tertiary amine and is, as a free base, lipide-soluble. Prostigmine is a methylated quaternary ammonium salt and lipide-insoluble. It appeared

likely that the inability of prostigmine to affect conduction, in contrast to eserine, must be attributed to the fact that the membranes surrounding the fibers are impervious to prostigmine. This has been tested in experiments on the giant axon of squid (37). The nerves were exposed to prostigmine in high concentration ( $10^{-3} M$ ). After the exposure, the axoplasm was extruded and the presence of prostigmine was tested. Whereas those enzyme inhibitors which affect the action potential were found in the axoplasm, prostigmine, in striking contrast, was completely absent, in spite of the thinness of the myelin sheath in the nerve preparation used.

This observation has considerable significance and has thrown new light upon the fundamental difference between axon and synapse. Acetylcholine and curare are, like prostigmine, methylated quaternary ammonium salts. The observation of Claude Bernard that curare acts exclusively on the neuromuscular junction, and does not affect nerve or muscle fiber, was for a century considered as evidence that the mechanism of transmission of the nerve impulse across that junction may differ fundamentally from that along nerve and muscle fibers. The excitability of synaptic junctions by acetylcholine was one of the two essential facts on which the hypothesis of neurohumoral or chemical mediation was based. The failure to effect conduction by acetylcholine even in extremely high concentrations (20 g./l.) was considered as evidence that the physiological function of acetylcholine is limited to synaptic junctions (118). But the failure of prostigmine to affect conduction, in contrast to all the other inhibitors of ACh esterase, and the limitation of its effect to the synaptic region indicate the existence of a structural barrier for methylated quaternary ammonium salts like acetylcholine, which, therefore, can reach the active surface only at the synaptic region.

In view of the importance of this question, it appeared desirable to have direct evidence that the axon surface membranes are impervious to acetylcholine. The problem has been investigated with the aid of acetylcholine labeled with isotopic nitrogen (189). Giant axons of squid were exposed to 20 g. acetylcholine labeled with  $N^{15}$ . After exposure, the amount of  $N^{15}$  in the extruded exoplasm was determined by means of a mass spectrometer and compared to the results obtained from a similar exposure to a tertiary amine labeled in the same way. The data obtained are summarized in Table IX. The tertiary amine penetrates easily and is nearly in equilibrium after 60 minutes. The amounts of  $N^{15}$  found after exposure to acetylcholine are negligible in spite of the high concentration used. 0.6 micromole nitrogen (8.6  $\mu$ g.)/g. axoplasm was found, which amounts to 0.67% of the nitrogen outside. Even assuming that this would be the nitrogen of the choline, no physiological effect can possibly be expected if the penetration proceeds at such a low rate. 1 g. of these nerves

can hydrolyze 2 to 4 mg. acetylcholine/hour and therefore the infinitely small traces penetrating at any given moment would be hydrolyzed before they could possibly accumulate to an effective concentration. Adding eserine would not change the situation because, in concentrations at which the ACh esterase would be inhibited to less than 80%, the rate of hydrolysis would be still much too high compared to the rate of penetration. Still higher concentrations of eserine would impair and finally abolish conduction even without penetration of acetylcholine from the outside.

However, even the negligible amounts of  $N^{15}$  found inside should not be considered as being part of a quaternary ammonium salt. They must be

TABLE IX  
PERMEABILITY OF MEMBRANES SURROUNDING STELLAR NERVE OF SQUID TO  $N^{15}$ -  
LABELED TRIMETHYLAMINE AND ACETYLCHOLINE

Com- ound <sup>a</sup>	Time of exposure, min.	N outside micro- moles/ ml.	Axoplasm extruded, mg.	Axoplasm total N, micro- moles	$N^{15}$ , atom per cent excess	N diffused into axo- plasm, micro- moles/g.	Penetra- tion, %
TMA	15	20	134.3	69.2	0.333	5.5	30.5
	25	20	78.8	40.5	0.425	7.1	39.5
	60	20	149.4	77.1	0.916	15.3	84.5
ACh	25	100	137.0	70.7	0.038	0.6	0.67 <sup>b</sup>

<sup>a</sup> TMA = trimethylamine, ACh = acetylcholine. Compounds contained 31 atom per cent excess  $N^{15}$ . After exposure of the nerves to the compounds, axoplasm extruded and  $N^{15}$  concentration determined.

<sup>b</sup> Even this negligible amount of N found inside must be attributed to impurities present in the ACh used. The 1430  $\mu$ g. N/ml. contained 55  $\mu$ g. nonquaternary N. The N which penetrated (8.6  $\mu$ g./g. or 0.6 micromoles) is therefore equivalent to 18% of nonquaternary N outside. This is in the range to be expected.

attributed to the impurities of nonquaternary nitrogen present in the acetylcholine. It was found that the alkali-distillable nitrogen in the acetylcholine bromide amounted to 0.6% by weight (3.8% of total nitrogen). This is equivalent to 55  $\mu$ g. nonquaternary nitrogen in the 1430  $\mu$ g. nitrogen/ml. solution used. The 8.6  $\mu$ g. nitrogen/g. axoplasm (or 0.67% of the outside concentration) must therefore be attributed to the nonquaternary impurities present in the acetylcholine used. The 8.6  $\mu$ g. nitrogen are equivalent to 18.0% of the nonquaternary nitrogen outside. The rate of penetration of trimethylamine was, for the same length of exposure, 39.5%. In view of the smaller gradient, the rate of penetration may have been actually smaller. The agreement between the amount

found and that to be expected on the basis of the impurities present is therefore entirely satisfactory. Fig. 40 shows the difference of the rate of penetration of the nitrogen of the two compounds.

The experiments show conclusively that the axon surface membranes are impervious to acetylcholine. They explain why the action of acetylcholine when applied externally is limited to synaptic junctions. The peculiar ability of the synapse to react to compounds which do not affect conduction appears thus to be a difference of structure. This is illustrated by the schematic presentation of the neuromuscular junction in Fig. 41. Only the compounds on the left side are capable of acting everywhere, because they may penetrate through the structural barrier which may be lipide,

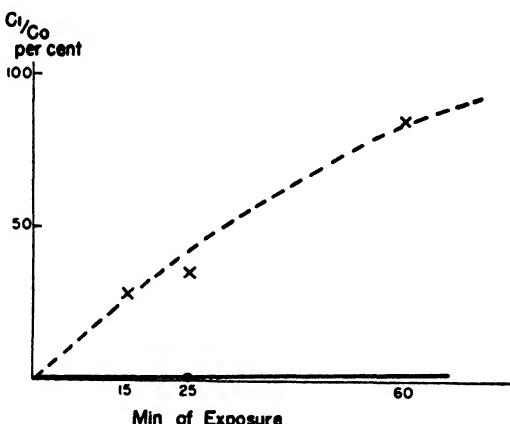


FIG. 40. Rate of penetration of trimethylamine and acetylcholine labelled with  $N^{15}$  into interior of giant axon of squid. Ratio of concentration of nitrogen of these compounds inside ( $C_i$ ) to that outside ( $C_o$ ) plotted against time of exposure. Dotted line indicates rate of penetration of N (X) on exposure to trimethylamine (286  $\mu$ g. nitrogen/ml.), the straight line, that of the nitrogen (●) found on exposure to acetylcholine (1430  $\mu$ g. nitrogen/ml. of which 55  $\mu$ g. was nonquaternary nitrogen).

although not necessarily so. In contrast, the compounds on the right side act only upon the postsynaptic membrane, which appears to be either less or not at all protected by lipide. Even the nerve ending of the presynaptic fiber, although not surrounded by myelin, appears to be covered by a lipide membrane according to the view of some neurohistologists. It is consistent with this view that the nerve ending seems to be inexcitable even by relatively high concentrations of acetylcholine in the perfusion fluid (31).

Even if a compound affects both the axon and the synapse, the concentration in each case may vary considerably. The unprotected active surface of the postsynaptic membrane will be affected by much smaller concentrations than those necessary for an action on the fiber. An inter-

esting illustration is furnished by the significant observations of Roeder and associates (184). These investigators found that DFP abolishes synaptic transmission in much lower concentrations than those which abolish axonal conduction. DFP is very lipide-soluble and may therefore accumulate in the myelin. As described above, the inside concentration of DFP, at the time when conduction disappears, is small compared to the outside concentration. It is obvious that the relatively high concentration of a drug affecting axonal conduction as compared to that affecting synaptic transmission must be explained by the necessity of penetrating the structural barrier.

It may be noted that the action of procaine and other anesthetics cannot be explained in terms of ACh esterase inhibition. These compounds are very weak inhibitors of ACh esterase although other esterases seem to be

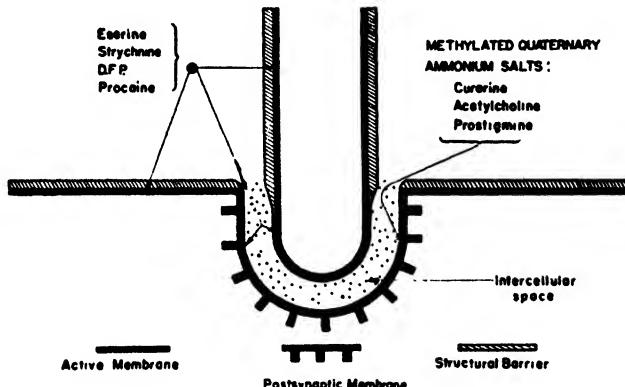


FIG. 41. Scheme of the neuromuscular junction.

affected more strongly (162). But, as pointed out by Thimann (199), these compounds are tertiary amines and have some resemblance in structure to acetylcholine. It may be that they act upon the same active center of the proteins of the membrane as the acetylcholine itself. Even if they are unable to change the condition of the protein they may block conduction by preventing acetylcholine from affecting the active center. In contrast to acetylcholine the local anesthetics will not be rapidly inactivated by ACh esterase. Only in those cases in which there is an ester in the molecule forming part of the active group, they may be hydrolyzed, but probably at a low rate.

#### B. RELEASE OF ACETYLCHOLINE DURING ACTIVITY

The excitability of the synapse by acetylcholine and the absence of a stimulating effect on the fiber do not justify, in view of the experiments

described, postulation of a role of acetylcholine limited to the synaptic junctions. The second fact on which the hypothesis of chemical mediation was built—the appearance of acetylcholine in the perfusion fluid—has also to be reconsidered. This appearance is obviously possible only there because of the absence of an insulating membrane. If the ester cannot pass through the barrier into the interior, it will not be able to leak from the interior to the outside in stimulated nerve and muscle fibers. But even at the synaptic junction, the ester does not appear under physiological conditions. It is an important fact, emphasized by Dale and associates, that the ester appeared in their experiments only if the normal mechanism responsible for the rapid removal of the ester, *viz.*, ACh esterase, is to a large degree inactivated by the presence of eserine. Even in presence of the drug, the amounts leaking out are infinitely small, one-hundred-thousandth to one-millionth of that required to set up a stimulus, a discrepancy not easily explained in terms of chemical mediation. In Loewi's original observations on the frog heart, no eserine was present but it may be recalled that considerable difficulties were encountered by him as well as other investigators when they tried to reproduce the appearance of the ester. For this reason, Loewi's hypothesis met considerable opposition and has been repeatedly criticized (5,172). In the heart preparation which has been perfused for a certain period of time with Ringer solution, the postsynaptic membrane may no longer be in a completely normal condition and thus permit leakage of the compound, which under physiological conditions is an intracellular metabolite and rapidly inactivated. The condition of the membrane may depend upon a variety of factors, such as length of the perfusion period, composition of the perfusion fluid, condition of the frog, species used, etc., and this may explain the difficulties of many investigators to reproduce the observation. The same consideration may be applied to the finding of Kibjakow (101), who, in 1932, described the appearance of acetylcholine in the perfusion fluid of the synaptic ganglion in the absence of eserine. His observations were questioned by Dale's school, but it is conceivable that, due to much less perfect perfusion techniques in Kibjakow's experiments, the active membrane suffered more damage and thus permitted the leakage of measurable traces. In summary, there is so far no conclusive evidence that the appearance of the ester outside the cell is a physiological event.

The release of acetylcholine assumes, however, a new aspect in the light of another series of pertinent observations, which, at the time of their presentation, did not find sufficient attention. In 1933, simultaneously with or even prior to the findings of Dale and associates that acetylcholine appears in the perfusion fluid of the sympathetic ganglion and of the neuromuscular junction, Calabro had shown that, following prolonged stimula-

tion of the rabbit vagus, an acetylcholinelike substance is released from the cut end into the surrounding fluid (38). Binet and Minz (25) found in 1934 that, from the cut end of stimulated nerves, a compound is released which increases the sensitivity of the leech muscle to acetylcholine. Calabro's findings were confirmed and extended by Bergami (23,24) and by Babsky (16) and Babsky and Kisliuk (17) to motor nerves. In 1937 von Muralt described a difference of the acetylcholine content between stimulated and unstimulated nerve fibers (133). Recognizing the possibility of a very rapid disappearance of the active ester, he had developed a very elegant technique: he "shot" the nerves into liquid air. When tested after a short period of extraction, the amount of acetylcholine was 0.20  $\mu\text{g./g.}$  in the stimulated as compared to 0.12  $\mu\text{g./g.}$  in the control nerve. Later, in a large series of experiments, the difference between stimulated and unstimulated fiber was found to be 0.09  $\mu\text{g./g.}$  (134,135). The difference between the two nerves disappears if extraction is continued for a longer period of time. There is, therefore, some uncertainty as to the interpretation, but it is conceivable that the acetylcholine released is present in a free form and diffuses therefore from the frozen tissue more rapidly into the extracting medium than that part of the acetylcholine bound to protein or lipoprotein. In a remarkable study, Brecht and Corsten found, in 1942, that even in sensory nerves acetylcholine is released at the cut ends if the nerves are stimulated (29). The amounts were smaller than those found by previous investigators with motor nerves. Brecht and Corsten succeeded in demonstrating this release by using a most sensitive method, *viz.*, the contraction of the frog lung in the presence of eserine. Thus, the release of acetylcholine has been demonstrated in parasympathetic, motor, and sensory nerve fibers. These observations parallel the findings that the enzymes forming and hydrolyzing acetylcholine are present in all types of nerves although in sensory fibers in lower concentrations, and that the inactivation of ACh esterase abolishes conduction in all fibers.

The observations support the assumption that there is no difference in principle between the release of acetylcholine at the synapse and in the axon except that in the latter the ester cannot pass through the structural barrier. They make it still more probable that this release is an intracellular process and that the appearance outside the cell at the synapse must be attributed either to the poisoning of the enzymatic mechanism normally preventing the leakage or to some other damage of the active surface where it is least protected and most vulnerable. Since, at the time when these observations were described, acetylcholine was considered to be a chemical mediator and since it is inconceivable to assume chemical transmission in the axon, it was difficult to integrate them with nervous function except in the cautious way proposed by von Muralt. He called acetylcholine an

"*Actionssubstanz*" in the sense that it may be important, like many other substances, for nerve activity in the axon as well as at the synapse. von Muralt's cautious definition was well justified at that time, since nothing was known about the high speed of the reaction, the effects of the ACh esterase inhibitors on conduction, and all the other facts known today, which had to be established before it became possible to assume a direct association of the ester with the generation of the electric currents propagating the impulse. In the light of the recent investigations, all these findings demonstrating the release of acetylcholine in the axon appear as relevant as those concerning the release in the synaptic junctions and require therefore a modification of the original interpretation.

Another *Actionssubstanz* may be mentioned briefly, namely, vitamin B<sub>1</sub>. The role of this vitamin in pyruvic acid oxidation and its special importance for the nerve tissue is well known. There are, however, reports describing pharmacological actions of this substance and its release during nerve activity in the fiber and at the ending (1,131,132,135). The question has therefore been raised whether this vitamin has some function in nerve activity in addition to that in metabolism. A series of interesting facts is described in the monographs quoted. Their interpretation is, however, on the basis of the available evidence, difficult, and more information is desirable before a definite view becomes possible.

The structural barrier for acetylcholine present in the fiber, and its absence at the synapse, may be considered the main reason that attention of many physiologists interested in this compound was focused for such a long time on the synapse only. Knowledge of this structural difference is of obvious importance for pharmacology and clinical medicine; it may be helpful in developing new compounds which, according to the effect desired, should act predominantly on the fiber or on the synaptic junction. But the existence of structural barriers makes difficult an interpretation of cellular function based essentially on the effects of compounds applied externally. This may account for the great obstacles encountered and the many contradictory reports when the two criteria of chemical mediation were applied to different types of synapses. The great variations of anatomical structure, the biochemical composition of the surrounding medium, and probably many other accessory conditions may be essential in determining the effect of acetylcholine when applied externally, although the physicochemical mechanism of the propagation of the nerve impulse may be the same everywhere. In view of the physicochemical properties of acetylcholine and similar methylated nitrogen compounds, the difficulties will become nearly insurmountable in the study of brain and spinal cord, which contain such large amounts of lipide. It is therefore not surprising that the painstaking efforts to demonstrate or to disprove the "cholinergic"

nature of synapses in brain and spinal cord have resulted in a most unsatisfactory and confusing picture.

A recent report may be analyzed. It is an instructive example of the way in which the "cholinergic" nature of a synapse is tested and chemical transmission for a special focus either accepted or discarded. In a series of experiments, frog spinal cord was exposed to eserine in  $10^{-4} M$  concentration and to prostigmine in  $3$  to  $6 \times 10^{-5} M$  concentrations. No effect on synaptic transmission in the cord was obtained (69,70). Assuming that the ACh esterase in the spinal cord, under the conditions of the experiment, was completely inactivated by the eserine, any role of acetylcholine in the synapse of the spinal cord was categorically excluded. The assumption that the ACh esterase was completely inactivated was not tested experimentally but based on findings reported in the literature concerning the concentration necessary for inhibiting this enzyme in solution or suspension. The complexity of the kinetics of ACh esterase inhibition has been discussed above. It has been described that, even in solution, the degree of inhibition depends upon a great variety of factors, such as concentration of the enzyme, the substrate, and the inhibitor, on the type of inhibitor used, its continuous destruction, and others. Even *in vitro*, the definition of the degree of inhibition is very difficult. But, in Eccles' type of experiments, it is necessary to take into account, in addition to the kinetics of the inhibition, the structural barriers, penetration rates, the excess of enzyme available, and the many other factors upon which the effect of drugs may depend when they are applied to cells or organs. In order to inactivate ACh esterase to a sufficiently high degree for obtaining interference with conduction in a single-fiber preparation, the giant axon of squid, a concentration of at least  $10^{-3} M$  eserine must be used. This preparation has a lipide layer only a few microns thick. The eserine is applied at a pH of 8.2 to 8.4, the pH of sea water. At this pH, a greater fraction of eserine is undissociated and penetrates therefore more readily as free base than at pH of 7.2 used in Eccles' experiments, at which the compound is much more dissociated. For obtaining the effect with frog sciatic nerve, much higher concentrations are necessary. The reason for the failure to obtain an effect in the spinal cord with the low concentrations used is then obvious. Probably only an extremely small fraction of ACh esterase was inactivated, or possibly none. If the ACh esterase is really completely inactivated, conduction is not only altered but abolished.

In contrast to the conflicting results obtained when the "cholinergic" nature of synapses, especially in brain, is tested by the usual criteria of chemical mediation, the approach by the study of the enzymes connected with acetylcholine metabolism and their correlation with function did not encounter comparable difficulties. All results obtained in this way clearly

indicate the generality of the role of acetylcholine in all nerve tissues, including that of brain and spinal cord: the high concentrations of the two enzymes metabolizing the ester, the functional significance of the enzymic system indicated by the coincidence of its appearance with the beginning of function during growth, and the effects of DFP which have shown that ACh esterase in brain is indispensable for life.

### C. BASIC SIMILARITY OF CONDUCTION ALONG AXON AND TRANSMISSION ACROSS SYNAPSE

During the last 10 years, extensive investigations have been made on the electrical characteristics of transmission across the natural and artificial synapse ("ephapse") by a great number of investigators, mainly by Arvanitaki (3,4), Bullock (33), Eccles (68), Granit and Skoglund (87,88, 192,193), and many others. From the various investigations, considerable evidence has accumulated for Erlanger's view that the events considered to be peculiar to the synapse may be reproduced in the axon. It has become increasingly clear from these investigations that the basic mechanism in both cases is the same. The data suggest that the propagating agent across the synapse is the flow of current. According to Eccles (68), impulses in a presynaptic nerve fiber generate a current which gives a diphasic effect on the synaptic region of the postsynaptic cell. This current produces initially an anodal focus with cathodal surround followed by a more intense cathodal focus with anodal surround. The cathodal focus sets up a local response from which a catelectrotonus spreads over the postsynaptic cell membrane. This catelectrotonus, the end plate potential, sets up a propagated impulse in the postsynaptic cell as soon as a certain threshold is reached. The sequence of events is similar to that observed on artificial synapses (ephapses), and on a single-unit preparation of the synapse, the squid stellate ganglion (33). All indications whether based on electric signs or on biochemical data favor the assumption that the mechanism of the transmission of impulses across synapses and conduction along axons and the role of acetylcholine in this mechanism are fundamentally the same. The propagating agent in both events appears to be the flow of current. But, the release and the removal of acetylcholine must be essential events in the change of the presynaptic membrane during the flow of current across the synaptic region and in the postsynaptic membrane during the degeneration of the end plate potential. It would be difficult to picture these currents as being different in nature from those in the axon. The high concentration of ACh esterase in the postsynaptic membrane demonstrated in the experiments on denervated muscle supports the assumption of a high rate of acetylcholine metabolism in this purely muscular element.

It has been pointed out that the discharge in electric tissue is homologous

to the end plate potential. Several observations on this tissue suggest that the release and removal of acetylcholine are essential events in generating the discharge. The implications of the direct proportionality between voltage developed and ACh esterase concentration in the organ of *Electrophorus electricus* have been discussed in Section III, A. The discovery of the extraordinarily high concentration of ACh esterase in electric tissue made possible the assumption that acetylcholine might be the agent producing the depolarization presumably occurring during the action potential. The possibility of a depolarizing action of acetylcholine has been considered by Dubuisson and Monnier (66) and Cowan (54). When the prerequisite for such a theory, namely, the high speed of destruction of the active agent, appeared established, Auger and Fessard, in 1938, tested the effect of eserine on the discharge of the electric tissue of *Torpedo marmorata* (7). As may be seen in Fig. 42, in the presence of eserine, the height of the

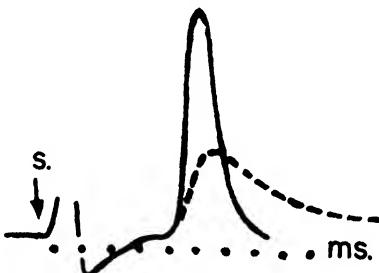


FIG. 42. Effect of eserine on the discharge of electric tissue of *Torpedo marmorata* (7). The fully drawn line shows the discharge in absence, the dotted line in presence, of eserine.

potential is markedly depressed and the duration of the descending phase considerably prolonged. This effect on the end plate potential is consistent with the assumption that the appearance and the removal of acetylcholine in the postsynaptic membrane may be essential for the generation of the potential.

In view of their corresponding biochemical and bioelectrical findings, Fessard and Nachmansohn decided then to test whether acetylcholine injected into the electric organ may produce an action potential. Such an electrogenic effect could be expected if acetylcholine is the compound responsible for the alterations of the membrane occurring during the discharge. In experiments carried out at Arcachon in 1939 on *Torpedo marmorata*, in which Fessard and Nachmansohn were joined by Feldberg, they were able to demonstrate that acetylcholine has an electrogenic effect (76,78,143). The arterial injection of acetylcholine caused potential changes similar to the natural discharge. However, the changes were small

and slow, and very large amounts were necessary for obtaining the effect. Fig. 43 illustrates the effects of acetylcholine injected in amounts varying between 5 and 200  $\mu$ g. 5  $\mu$ g. had no effect; with 200  $\mu$ g. the potential difference amounted to about 0.7 mv. and the descending phase had not yet reached the baseline after several seconds.

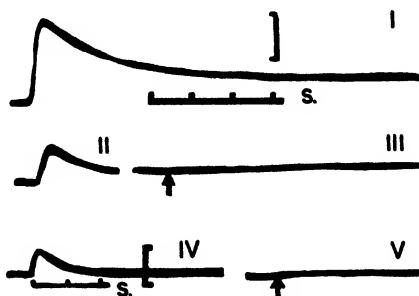


FIG. 43. Potential changes produced by intra-arterial injection of acetylcholine into electric organ of *Torpedo marmorata*. I, II, IV, and V correspond to injection of 200, 100, 20 and 5  $\mu$ g. ester; at III only perfusion fluid was injected. Between II and III sensitivity increased fourfold. 0.5 v. indicated at I, 0.1 v. at IV. Time in seconds.

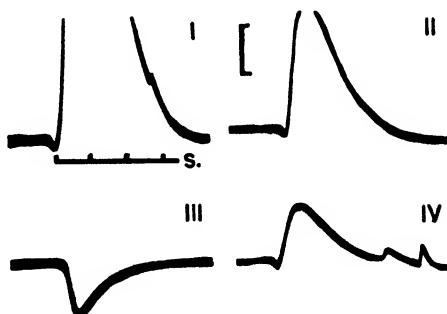


FIG. 44. Potential changes produced as in Fig. 43 but in presence of eserine. I, II, and IV correspond to injection of 10, 5, and 2.5  $\mu$ g. acetylcholine; at III only perfusion fluid injected. 0.5 v. indicated at II. Time in seconds.

If acetylcholine is injected in the presence of eserine, preventing a too rapid destruction of the ester, the effects are considerably enhanced. Fig. 44 shows that, under these conditions, an effect may be obtained even with 2.5  $\mu$ g. acetylcholine. With 10  $\mu$ g. the potential change produced is greater than 3 mv., although the duration is still about 3 seconds.

The experiments show that the ester may produce an alteration of the membrane preceding the flow of current. But although the potential changes resemble the normal discharge, there is a most striking contrast in

two respects: the small voltage and the long duration. The normal discharge occurs in 2 to 3 milliseconds; the voltage of a single unit is about 100 mv. Although a quantitative evaluation is impossible, since the number of units in series reached by the intra-arterial injection is unknown, the discrepancy as to duration and strength is enormous even in the presence of eserine. It is true that the method used is crude compared to an effect which might be expected if the compound were released from the nerve ending. In that case it would reach the opposite surface much faster. But in view of the large amounts injected, of which apparently a fraction at least reached the units, the smallness of the response is beyond all proportion. It thus becomes difficult to conceive that physiologically the substance is released from the nerve ending and, penetrating through the intercellular space, produces the end plate potential. This difficulty does not arise if it is assumed that the release of the ester is an intracellular event occurring in the postsynaptic membrane and produced by the flow of current. If only a few Å's separate the site of the release of the ester from that of its action, the effect may be extremely fast and powerful compared with any event which involves diffusion into the cell from the extracellular spaces.

If locally supplied energy is necessary for the small electric currents which propagate the impulse along the axon as postulated by Lucas and Adrian, it appears almost certain that such energy will be required for the generation of a potential in the second unit. The flow of current reaching the postsynaptic membrane may result in a release of acetylcholine which may act as a trigger in the chain of events and supply the energy for building up the end plate potential. It is remarkable that exactly this mode of action has been proposed by Lapicque (104) in 1936:

"l'état d'excitation suscité dans la sole nucléé peut y déclencher une réaction auxiliaire venant fournir le supplément de puissance requise. Tel serait le rôle de l'acetylcholine; c'est exactement le rôle que joue l'amorce dans la technique des explosifs . . . La production de l'acetylcholine serait, dans cette conception, située, non entre le nerf et le muscle, mais dans le muscle lui-même, auquel appartient sans conteste la sole nucléé. Il s'agirait donc strictement parlant, non d'un intermédiaire dans la transmission de l'excitation entre nerf et muscle, mais d'un premier stade, formant relais dans l'excitation musculaire pour assurer sa généralisation à toute la masse du myome."

The possibility of demonstrating an electrogenic effect of acetylcholine in electric tissue is again due to the fact that the postsynaptic membrane is not protected by a structural membrane. It may be mentioned in this connection that Auger and Fessard (7) have definitely established the effect of curare in the electric tissue homologous to that known for the end plate. The contradictory results of previous observers must apparently

again be attributed to the impermeability of surrounding structures. The curare, being a methylated quaternary ammonium salt, may have an effect on the protein or lipoprotein of the active membrane similar to that of acetylcholine itself. But, in contrast to the ester, curare cannot be removed by hydrolysis, and this may be the reason that the effect persists until the drug is removed by diffusion. The flow of current arriving from the presynaptic fiber may still release acetylcholine in the membrane from its bound form but, as long as the curare action lasts, the ester cannot have any effect except in incomplete "curarization," where the release of acetylcholine may still have an effect; it will, however, be smaller since the membrane is not in normal resting condition.

The effect of eserine described by Auger and Fessard (Fig. 42) resembles indeed that obtained with partial curarization of the end plate. The depression and prolongation of the potential in the presence of eserine must be attributed to the persistence of acetylcholine which is released in the membrane and cannot be removed with the usual speed. With still higher concentrations of eserine, a complete "curarization" will be obtained. Thus, the two methylated quaternary ammonium salts appear to have similar effects. With massive doses of acetylcholine, it is even possible to produce a "curarelike" effect without eserine. In that case, acetylcholine can obviously not be removed by ACh esterase with sufficiently high speed from the site of action even in absence of eserine. The possibility of a role of acetylcholine in the generation of bioelectric currents has been discussed by Koshtojanz in a series of papers (102a). Similar views have been developed by Gesell, who has based his theory on the electrogenic action of acetylcholine at the site of its liberation (83,118a).

As pointed out by Erlanger, conduction along the axon and transmission across synapses may vary as to quantitative aspects to some extent. This is not surprising in view of the discontinuity and other structural differences. Although the time relations are similar, there is a synaptic delay of the order of a millisecond. This may be the result of several factors, *e.g.*, the decreased diameter of the nerve fiber near the ending, which may lead to a decreased rate of conduction. Exact measurements of these various factors are difficult due to obvious technical reasons. But the quantitative differences between intracellular and transsynaptic propagation are well in the expected range and none of them requires the assumption of a fundamentally different mechanism. Much more convincing evidence than that available today would be necessary for the acceptance of the idea that acetylcholine assumes at the synapse a function entirely different from that in the axon, *i.e.*, is released from the nerve ending, penetrates the neuroglia, and acts on the postsynaptic membrane, thus substituting the flow of current by chemical mediation. The interpretation proposed harmonizes the

so-called electrical and chemical concepts of synaptic transmission and integrates the progress achieved concerning the structure, the biochemical data, and the electrical signs of nerve activity.

### VII. Possible Mode of Action of Acetylcholine

Although the necessity of acetylcholine for conduction has been conclusively established, the precise function of the ester is unknown. A possible mode of action may be briefly discussed.

The basis of all modern concepts of conduction is the membrane theory. Essentially, this theory assumes that the nerve is surrounded by a polarized membrane, the positive charge being on the outside and the negative on the inside surface of that membrane. The concentration of potassium in the interior is about 20 to 30 times as high as in the outside fluid whereas the sodium concentration is much higher outside. Differences in ionic concentration have always been considered as the source of the electromotive force. The polarization has been attributed to the fact that, in resting condition, the membrane is permeable to potassium ions only. These ions in penetrating the membrane charge the outside positively, but the negative ions, unable to pass the membrane, are kept back on the inside surface and charge it negatively.

During activity, a wave of negativity sweeps down the fiber. The process was pictured in the following way: if a stimulus reaches the surface membrane, a depolarization occurs which was attributed to an increased permeability of the membrane for the negative ions. The depolarized point becomes negative to the adjacent region and flow of current will result. These local currents, the "*Stroemchen*" of Hermann, stimulate the adjacent point which in its turn becomes depolarized and produces thus another local current. The process is successively repeated and, in this way, the nerve impulse is propagated along the axon.

During the last 10 years, however, considerable modifications of the original theory became necessary (89). It has been shown by Hodgkin and Huxley (93,94) and Curtis and Cole (58) that there is not only a depolarization of the membrane during the passage of the impulse but an actual reversal of the charge. The spike potentials were found to be greater than the potential differences in rest and, in some cases, nearly twice as great. It is clear from these observations that, during activity, the potential developed across the membrane is not merely the abolition of the resting potential due to a transient breakdown of the polarized cell membrane. The events are much more complex.

Recent observations of Hodgkin and Huxley (95) indicate that, during activity, potassium ions leak out from the inside of the axon and sodium ions move in. An exchange of sodium and potassium across the membrane

apparently also occurs in the resting condition, as has been shown by Rothenberg and Feld (187) with the giant axon of squid by means of radioactive isotopes.

These investigators exposed the axon to artificial sea water in which all or part of either sodium or potassium were replaced by the corresponding radioactive ions. They found that within 20 to 30 minutes nearly all of the sodium inside was exchanged against the radioactive sodium outside.

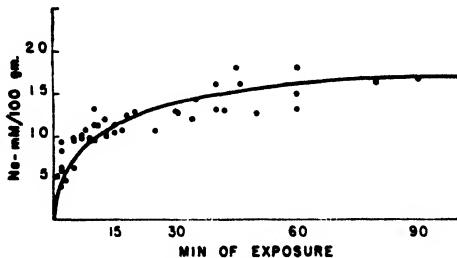


FIG. 45. Na penetration across the membranes of the giant axon of squid when exposed to artificial sea water containing either 0.13 M or 0.065 M  $\text{Na}^{24}\text{Cl}$ . Total NaCl concentration is 0.52 M. The penetration of Na in millimoles (mM)/100 gm. axoplasm (wet weight) is plotted against time of exposure in minutes.

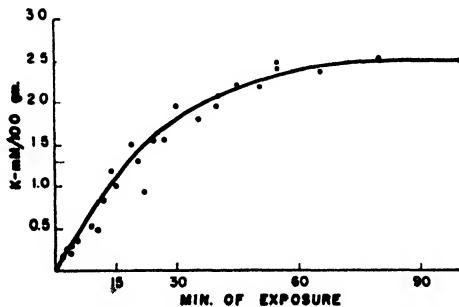


FIG. 46. K penetration across the membranes of the giant axon of squid when exposed to 0.013  $\text{K}^{42}\text{Cl}$  in artificial sea water. The horizontal broken line on the ordinate indicates the  $\text{K}^{42}$  concentration outside. The penetration of  $\text{K}^{42}$  in millimoles (mM)/100 gm. axoplasm (wet weight) is plotted against time in minutes.

In case of the potassium only a fraction of the inside concentration, about 10%, were exchanged. The results are summarized in Figs. 45 and 46. The experiments show that the concentration gradient between inside and outside is a dynamic and not a static equilibrium. The values for the temperature coefficient,  $Q_{10}$ , for the potassium and sodium exchanged were close to 1.25, the value calculated theoretically from ionic conductivity measurements. These figures do not support the assumption that im-

portant energy yielding reactions are involved in the transport of ions across the axonal membranes in resting condition.

During activity about  $4.5 \times 10^{-12}$  mole sodium were found to penetrate into the nerve per impulse per square centimeter surface (86 and 189a). This figure is of the same order of magnitude as that estimated by Hodgkin and Huxley for the leakage of potassium. On the basis of measurements of conduction change, they estimated that  $1.7 \times 10^{-12}$  mole potassium/impulse/cm.<sup>2</sup> surface leaks out, using a single axon of *Carcinus maenus*. Their estimations were confirmed by the observations of Keynes (100). He soaked *Carcinus* nerves in Ringer solution containing radioactive potassium and compared the leakage in rest and during stimulation. The amount of potassium lost during stimulation was calculated to be  $2.1 \times 10^{-12}$  mole/cm.<sup>2</sup> surface/impulse. Considering the difference of methods and material used in the two laboratories, the results may be considered to be in satisfactory agreement.

The understanding of the mechanism by which the small electric currents are propagated along the axon requires then the understanding of the reactions which change the permeability of the membrane and make the rapid flow of ions possible, as was predicted by Ostwald in 1890 (176). That such a change of permeability occurs has been definitely established by measurements by Cole and Curtis of the impedance changes during activity in the single nerve fiber preparation of the squid. These investigators measured the impedance changes with alternating current of varying frequency applied across a nerve fiber (46). The impedance was always reduced during the passage of the impulse. Analyzing their results, they estimated that the membrane resistance breaks down during activity from about 1000 ohms/cm.<sup>2</sup> to about 40 ohms/cm.<sup>2</sup>

For the understanding of bioelectric currents it is necessary to explain the change of membrane resistance in molecular, *i.e.*, in chemical or physico-chemical terms. The process leading to increased permeability for ions probably involves changes of protein or lipoprotein. A really satisfactory answer to the problem of the role of acetylcholine in conduction will become possible if the precise function of the ester in this process can be determined. All that may be said at the present state of knowledge is that a compound which may be metabolized in a few microseconds may well account for such fast transitory changes. The local currents, the "Stroemchen," may lead to a release of acetylcholine from a bound to a free form. This may be the primary event responsible for the change of the protein or lipoprotein which increases the permeability to ions and thereby generates a new "Stroemchen." Several other facts are consistent with this assumption, such as the necessity of the rapid removal of the ester without which conduction becomes impossible, the ability of the ester to produce potential

changes, and the direct proportionality between the potential changes and the potential rate of the enzyme removal. Whatever the final answer may be, the hypothesis has proved its usefulness, since it has been and continues to be helpful in finding new facts.

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## CHAPTER IX

### Chemical Control of Nervous Activity

#### B. Adrenaline and Sympathin

By H. BLASCHKO

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The common origin of the chromaffin cells and the nerve cells of sympathetic ganglia has been known for a long time, but only the discovery by Loewi (92) of the "accelerans substance" of the frog heart in 1921 led to

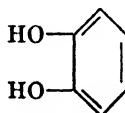
the full recognition of the functional equivalence of these two cell types. The adrenergic cell is today considered a "chromaffin" cell with its secretory properties banished to its extreme processes, an arrangement which ensures the localization of the response in the excitable tissue to an area innervated by neurons which happen to be stimulated. This somewhat crude picture may serve as a basis for discussion in the following review.

## I. Assay of Adrenaline and Related Amines

Until recently the levorotatory form of adrenaline was the only sympathomimetic amine isolated from vertebrate tissue and chemically identified. Often, however, the amount of adrenaline present in biological material has been too small for the chemical method of identification to be used and adrenaline had to be assayed and characterized by methods which will work with smaller amounts of material.

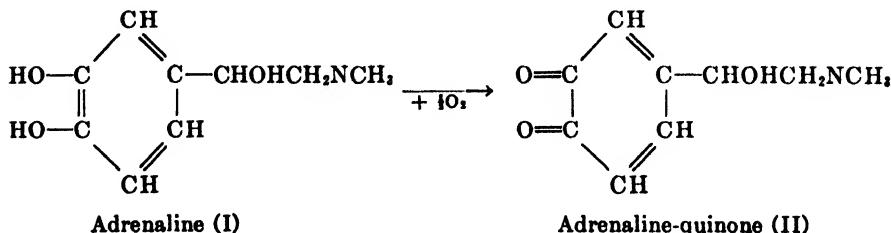
## A. CHEMICAL METHODS OF ASSAY

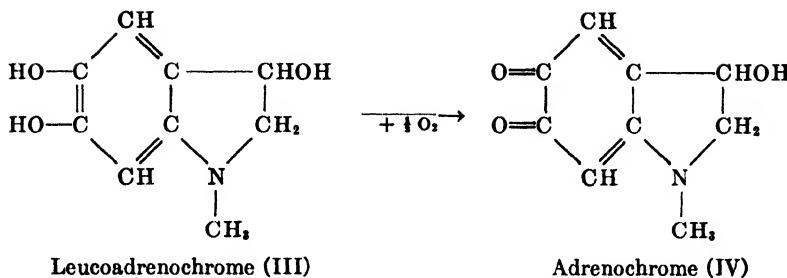
These are less certain than the biological methods discussed below. They all depend on the catechol grouping present in the adrenaline molecule. Since this group is also found in related amines it is usually not possible, or not easy, to distinguish adrenaline chemically from closely related substances. A useful method is that described by Gaddum and Schild (55),



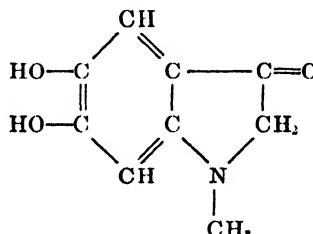
## Catechol

which is based on the fluorescence which develops when an adrenaline solution is made alkaline. Adrenaline is oxidized with the formation of an indole derivative. This compound is called adrenochrome (IV); it has been obtained pure and as a crystalline product (63). The probable steps in the formation of adrenochrome from adrenaline are shown below:

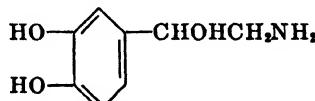




Adrenochrome itself is not fluorescent; some authors believe that the fluorescent material is leuocadrenochrome (135, 140), but that is not generally accepted. According to Lund (94b), the fluorescent substance can be prepared not only from adrenaline, but also from adrenochrome, in the absence of oxygen. He suggests that no change in the degree of oxidation takes place in the conversion of (IV) to the fluorescent material. This is in agreement with findings of Harley-Mason (65b). Lund attributes this structure to the fluorescent substance:

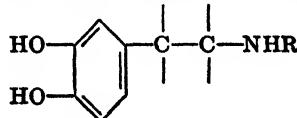


The fluorescence reaction is useful, as it is more sensitive for adrenaline than for closely related amines; *e.g.*, the corresponding primary amine, noradrenaline (also called arterenol), does not give a fluorescence as rapidly and as strongly as adrenaline itself. West (141) has recently re-examined the Gaddum and Schild test; he finds that the ratio of equiactive amounts of noradrenaline to adrenaline is 33 to 1.

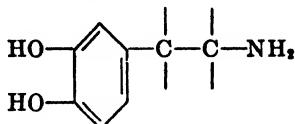


Noradrenaline

The difference of the two amines in the fluorescence test is probably related to the fact that secondary amines of the general structure:



seem to react more readily to form indole derivatives of the adrenochrome type (III) than the corresponding primary amines (32,35,47):



Another method, based on the reducing properties of adrenaline, makes use of the blue color given by adrenaline when added to arsenomolybdic acid in the presence of sulfite (143). Shaw (124) has worked out a modification of this method, which is not specific for adrenaline, but the fivefold increase in the color intensity on adding alkali is fairly specific and not given by noradrenaline. West (141) finds that in this "specific Shaw test" adrenaline is sixteen times as active as noradrenaline.

Euler and Hamberg (45e) describe a method based on the differences in the rates of formation of the iodochromes of adrenaline and noradrenaline. (For the structure of the iodochrome of adrenaline see page 607). Other methods that allow the determination of noradrenaline in the presence of adrenaline have also been recently described (1a; see also 122a).

The first satisfactory separation of noradrenaline and adrenaline was achieved by James (79b); he used paper chromatography, with phenol as solvent. This method has since been used by other workers with success. A second method has been briefly described by Bergström, Euler, and Hamberg (9a): they have separated the two bases by countercurrent distribution between 0.02 N HCl and phenol.

## B. BIOLOGICAL METHODS

Pharmacologically active substances of similar chemical structure can often be distinguished when they show quantitative or qualitative differences in their effects on excitable tissues. When a variety of biological tests is used for the assay of one sample the results obtained with different tests often disagree when the active principle in the sample differs from the substance against which it is assayed. For adrenaline, the basis of such a comparative method of assay was given by the work of Barger and Dale (9) in 1910, when the term "sympathomimetic amine" was introduced. It was shown that substances chemically related to adrenaline were more or less perfect sympathomimetics; they shared some properties with adrenaline, but there were quantitative and qualitative differences. Since 1910 much work on this group of substances has been done and new sympathomimetic amines continue to be added to the list of the already existing ones. Many of these substances are chiefly of interest to the pharmacologist, but some may normally occur in the body, and of these noradrenaline has recently become particularly interesting.

Noradrenaline has usually been studied as the racemate, but this has been resolved into its optically active components; the levorotatory substance is much more active in various tests than its dextrorotatory stereoisomer (24, 132, 94a). In its pharmacological actions it is on the whole adrenalinelike, but there are certain differences. Barger and Dale found that adrenaline relaxed the nonpregnant uterus of the cat, whereas noradrenaline was without this action. There are other differences; for instance, the pressor action is not reversed by doses of ergotoxine which reverse the effect of adrenaline. As to qualitative differences, it has long been known that noradrenaline is more active on the arterial blood pressure

TABLE I

RATIO OF DOSE OF DL-NORADRENALINE TO EQUIACTIVE DOSE OF L-ADRENALINE ON VARIOUS TEST PREPARATIONS (141)

Test object	Ratio	Excitor (E) or inhibitor (I) action
Cat, blood pressure . . . . .	0.8	E
Cat, pregnant uterus . . . . .	0.8	E
Cat, ileum . . . . .	1.0	I
Cat, nictitating membrane . . . . .	1.25	E
Rabbit, ileum . . . . .	2.0	I
Rat, ileum . . . . .	3.0	I
Rabbit, pregnant uterus	4.0	E
Rabbit, nonpregnant uterus	5.0	E
Frog, blood vessels . . . . .	5.0	E
Frog, Straub heart . . . . .	8.0	E
Cat, nonpregnant uterus . . . . .	10.0	I
Frog, perfused heart . . . . .	33.0	E
Rat, nonpregnant uterus . . . . .	100.0	I

of the cat than adrenaline. West (141) has compared the action of the two substances on a number of tests; we reproduce here a table from his paper giving equiactive doses of DL-noradrenaline and L-adrenaline; this shows many quantitative differences between the two amines. In most tests noradrenaline is less active than adrenaline, irrespective of whether the drugs cause excitation or inhibition. The rat uterus is especially insensitive to noradrenaline; this preparation is therefore particularly useful for the assay of adrenaline in the presence of noradrenaline. The isolated rat's uterus is suspended in a medium low in Ca and at a low temperature, as recommended by Jalon, Bayo, and Jalon (79a); under these conditions the spontaneous activity of the organ is suppressed. The muscle is made to contract—acetylcholine or another suitable stimulant is applied—and the depression of contraction by adrenaline is measured.

In a recent study of biological test methods for adrenaline and related amines in blood, Gaddum, Peart, and Vogt (54a) have recommended the use of five methods for the quantitative assay: the rat's uterus and colon, the rabbit's ear, the cat's spleen, and the cat's nictitating membrane; the authors describe methods by which interferences due to the presence of other substances in the blood can be avoided.

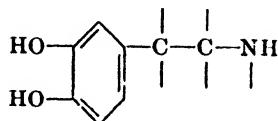
Bacq and Fischer (6) distinguish between adrenaline and noradrenaline by recording simultaneously the nonpregnant uterus and the nictitating membrane of the cat; both amines cause a contraction of the nictitating membrane, but only adrenaline will cause a relaxation of the uterus. Bülbring and Burn (26a) have made use of the different sensitivity of the normal and the denervated nictitating membrane of the spinal cat to the two amines: the ratio of the contraction of the denervated membrane to that of the normal membrane is greater for noradrenaline than for adrenaline; this enables the determination of the relative amounts of the two amines when both are present.

The work of Euler and his colleagues will be discussed more fully below; they have used for the assay the cat's arterial blood pressure, the cat's uterus *in situ* and the hen's rectal cecum (45c).

## II. Chromaffin Tissues

### A. NATURE OF THE CHROMAFFIN REACTION

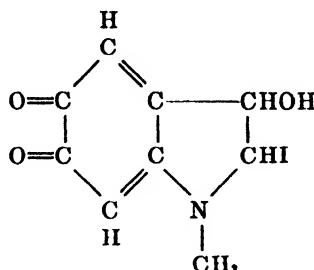
The term "chromaffin reaction" is really a misnomer; potassium bichromate acts by oxidizing the diphenol adrenaline with the appearance of a colored oxidation product. The reaction is probably not specific for adrenaline; *in vitro*, it is shared by other substances which contain the grouping:



Bichromate is not the only oxidizing agent that will produce such a color reaction; Szent-Györgyi (131) has shown that potassium iodate, a colorless substance known to give a color reaction with adrenaline, will produce a similar reaction in the suprarenal medulla. Iodate can also be used to demonstrate "chromaffin" tissue histologically (89).

The oxidation of adrenaline by potassium bichromate does not lead to a chemically defined oxidation product (60), but the substance formed when adrenaline is oxidized by potassium iodate has been isolated in crystalline

form (119); the following structure is attributed to this compound, which is called the iodochrome of adrenaline:



It is not known if other adrenalinelike substances, *e.g.*, noradrenaline, also give a "chromaffin" reaction; it seems possible that in these oxidation reactions the secondary amines like adrenaline react more readily than the primary amines (32,47), but this question has not been systematically studied.

## B. DISTRIBUTION OF CHROMAFFIN TISSUE

### 1. Endocrine Tissue

Chromaffin tissue has been found in all Chordata except *Amphioxus*; among invertebrates its distribution seems to be less general (see the recent review by Bacq, 4). An interesting case is that of annelid worms. Here the chromaffin reaction is given by certain ganglion cells (57,113,115). Gaskell's experiments suggested that these cells send out nerve fibers to muscles which respond to the stimulation by way of the nerve as they do to adrenaline; these are the muscles of the vascular system and alimentary tract. For the alimentary tract, an antagonistic action of adrenaline and acetylcholine has been described (103). The similarity of this type of nervous system to the autonomic nervous system of vertebrates has repeatedly been noted (59,108).

The substance found in the chromaffin ganglion cells of the earthworm is adrenalinelike in action (12); in the leech, Gaskell (58) showed that an extract of the ganglia caused relaxation of the virgin uterus of the cat. In the same ganglia Bacq and Godeaux have recently found a substance said to be pharmacologically indistinguishable from L-adrenaline (7). There have so far been no reports of the occurrence in annelids of other sympathomimetic amines, *e.g.*, noradrenaline.

In vertebrates the chromaffin reaction is given by the cells of the suprarenal medulla, by the paraganglia and by cells embedded, either in clusters or isolated, in various parts of the sympathetic system (85). The paraganglia are particularly well developed in immature animals; in mammals,

they undergo involution during development. In man, they have almost disappeared by the time of puberty. They contain adrenalinelike active material, probably adrenaline itself or a closely related substance (13,34). They secrete this material just as do the cells of the suprarenal medulla; after a dose of insulin their adrenaline content and their chromaffin reaction are reduced, as in the medullary cells (81,114).

Unlike the paraganglia, the isolated chromaffin cells of sympathetic ganglia seem to persist in many animals. In the cat solar plexus they are innervated by thinly medullated nerve fibers (112). Blotevogel (21-23) has made a study of the chromaffin cells in the cervical uterine ganglion of the white mouse; he finds in mature animals a fairly constant ratio of chromaffin cells to ganglion cells of 3.4%; this figure is reduced to about 0.9% in ovariectomized animals; in pregnancy the ratio increases. These cells also occur in the cat, *e.g.*, in the superior cervical ganglion. In man, they undergo a reduction in number during growth; they were still numerous in the cervical uterine ganglion of a girl aged 12 years (111). They have been seen in the human adult (79,130,139,144). According to Stöhr, the chromaffin cells form part of the syncytium surrounding the sympathetic ganglion cells, but this is not generally accepted (70).

The chromaffin cells of the sympathetic ganglia are of interest in connection with recent work on the transmission of nervous impulses across ganglionic synapses in the sympathetic nervous system. Kibjakow (82) had found in the perfused superior cervical ganglion of the cat that the perfusion fluid collected during the stimulation of the preganglionic fibers, when reinjected into the ganglion, lowered the threshold for preganglionic stimulation. Bülbring and Burn (26) showed that in small doses adrenaline improved the transmission across the ganglionic synapse, and they suggested that the chromaffin cells present in the ganglion were the source of the active substance in Kibjakow's experiments. This suggestion was further strengthened by Bülbring (25), who found that during preganglionic stimulation the perfusate contained an adrenalinelike substance which was characterized by its actions on the frog heart and on the pigeon rectum as well as by the fluorescence reaction of Gaddum and Schild (see also 27a).

Adrenalinelike actions of large doses of acetylcholine on the mammalian heart, especially after atropine, which could be abolished by ergotamine, have recently been described (71,95,96). These effects were also found to be abolished after large doses of nicotine. It has been suggested that chromaffin cells present in the heart are responsible for these effects (71); these cells release adrenaline under conditions comparable to those which lead to a release of adrenaline from the suprarenal gland. Chromaffin cells have been described in the heart of a number of mammals, including the kitten (133).

Tumors of the chromaffin tissue are known in man as pheochromocytoma; these may take their origin either in the suprarenal medulla or in extramedullary chromaffin tissue; the latter are also known as paraganglioma. These tumors have been considered rare, but the recent clinical literature suggests that they are more common than used to be believed. The clinical symptoms leave no doubt that an active sympathomimetic amine is released from the chromaffin tissue of these tumors. Interest in these tumors has recently been revived when it was shown that they contain noradrenaline (73a; see also 61a).

The chemical nature of the chromaffin material will be more fully discussed in connection with the biosynthesis of adrenaline (Section IV (page 623)).

## 2. Exocrine Tissue

Only one instance of exocrine chromaffin tissue is known: the skin glands ("parotid gland") of certain tropical toads (see review by Gessner, 61). Abel and Macht (1) were the first to isolate and identify L-adrenaline from the secretion product. The secreting cells show the typical chromaffin reaction with bichromate. According to Bacq and Lecomte (8), denervation of the gland in *Bufo arenarum H.* lowers the adrenaline content of the secretion product from 2.5–3% of the dry weight to about half this value. Diazotation of the secretion gives rise to a color which is partly due to adrenaline, partly due to other phenolic substances. The excretion of phenols other than adrenaline is increased after denervation. Nothing is known about the occurrence of sympathomimetic amines other than adrenaline in the gland.

## III. The Adrenergic System

The occurrence of chromaffin material in the nerve cells of annelid worms is of particular interest in connection with the function of "adrenergic" neurons. In his book *The Involuntary Nervous System*, published posthumously in 1916 (59), Gaskell, commenting on his son's observations, writes:

"In the case of the medullary cells of the suprarenal gland, the adrenaline is discharged from the cell into the surrounding fluid by the action of fibers of the splanchnic nerve; it is just possible that in the case of the leech the adrenaline passes from the cell to the periphery by way of the motor nerve itself. If such a suggestion prove to be true it opens out a new and most important chapter in our conceptions of the nature of nervous action."

These words were written before transmitter theories were generally accepted, but already in 1904 Elliott had discussed the possibility that in the sympathetic nervous system "adrenaline might be the chemical stimu-

lant liberated on each occasion when the impulse arrives at the periphery" (38). Later, in 1913, however, Elliott (39) had modified his views. He compares the functions of the ganglion cell and the paraganglion cell (the term "paraganglion cell" is used by Elliott for all chromaffin cells, in the paraganglia, in the suprarenal medulla, and elsewhere) and writes: "their present anatomical separation may be the index of a separation of functions which were once held by the two in common, when the adrenaline liberation was a part of the nervous impulse and the paraganglion cell a part of the ganglion cell."

By 1913 the original concept of 1904 had thus undergone a change: the liberation of adrenaline as a part of the nervous impulse in vertebrates is considered a thing of the phylogenetic past. In the intervening period, the earlier theory had been criticized by Barger and Dale (9). One of the reasons these authors did not accept Elliott's theory is set down thus:

"The conception of sympathetic nerve-impulses as acting by the liberation of adrenaline [adrenaline] seems to us unsatisfactory for another reason. It involves the assumption of a stricter parallelism between the two actions than actually exists. Adrenine has, in common with the other methylamino bases of the catechol group, the property of exaggerating inhibitor as compared with motor effects. The inhibitor effects of these methylamino bases are relatively prominent not only as compared with those of homologous bases, in particular the amino-bases, but also with those of sympathetic nerves." And they continue: "The action of some of the other bases, particularly the amino- and ethylamino-bases of the catechol group, corresponds more closely with that of sympathetic nerves than does that of adrenine."

The amino bases studied by Barger and Dale included noradrenaline. In the following, recent work will be discussed which shows that noradrenaline occurs in the body.

#### A. NATURE OF THE SYMPATHETIC TRANSMITTER

In his earlier work Loewi showed that the "accelerans substance" of the frog heart was adrenalinelike (92,94) and he later gave data which show that it is probably identical with L-adrenaline; he compared its pharmacological action on the frog heart to the fluorescence it gave in the Gaddum and Schild test; the adrenaline equivalents obtained with both methods gave results which were in reasonably good agreement (93). Extracts of the frog heart contained a substance with similar properties.

Doubts were thrown on the identity with adrenaline of the substance circulating in the blood stream after stimulation of the sympathetic nerves by the work of Cannon and his colleagues. This work is reviewed in the book *Autonomic Neuro-Effectors* by Cannon and Rosenblueth (30). Cannon introduced the term "sympathin" for this substance. It is not in-

tended here to discuss fully the interpretation given by Cannon to his observations. He stimulated the sympathetic nerves of cats after removal of the suprarenal glands and recorded the responses of distant organs—often denervated organs—to the substance released into the blood stream. Most of these effects were identical with those observed after an injection of adrenaline, but certain differences were seen when the nerves stimulated were the sympathetic supply to the liver. The active material released into the circulation under these conditions is now usually called liver sympathin; it differed from adrenaline principally in these points: (1) It had little or no dilator action on the iris. (2) It had no inhibitory action on the nonpregnant uterus. (3) It caused a rise of blood pressure which was not reversed by ergotoxine.

Cannon thought that the mediator was in fact adrenaline, and that the substance which found its way into the blood stream was not the mediator itself, but a product of the mediator and the effector cell; this product was sympathin. Sympathin derived from an effector organ which had been excited by the stimulation was different from the sympathin that was formed in an effector organ that had been inhibited as a result of sympathetic stimulation. There were accordingly two substances, sympathin E and sympathin I, and these were assumed to be present in the circulation in varying proportions depending on the type of response elicited by the stimulation, which was usually a mixture of excitatory and inhibitory reactions. Liver sympathin was identified with sympathin E.

Liver sympathin has been the subject of much experimental study and Cannon's experiments have been confirmed. In 1934, Bacq (3) had suggested that sympathin E might be identical with noradrenaline. Experimental work on the comparison of liver sympathin and noradrenaline began with an observation by Stehle and Ellsworth (129), who pointed out that after a dose of ergotoxine the effect of noradrenaline on the arterial blood pressure of the cat was a rise, while that of adrenaline was a fall in blood pressure; noradrenaline resembled in its action that of liver sympathin.

Melville (102) studied the action of two benzodioxan derivatives synthesized by Fourneau, 883 F (diethylaminomethylbenzodioxan) and 933 F (piperidylmethylbenzodioxan), on the blood pressure response to splanchnic stimulation (with the suprarenal glands excluded from the circulation) and compared this action of the two compounds to their effect on the response to both adrenaline and noradrenaline. The rise of blood pressure after splanchnic stimulation was found not to be reversed by 883 F and 933 F. The pressor effect of adrenaline was reversed, but that of noradrenaline, although slightly reduced, was not reversed.

Greer, Pinkston, Baxter, and Brannon (64) studied the effects of stimulation of the hepatic sympathetic nerves in comparison to those of adren-

aline and noradrenaline. They observed the effects on the arterial blood pressure, the uterus, the nictitating membrane, and the iris. They came to the conclusion that the actions of both noradrenaline and liver sympathin differed from those of adrenaline in essentially the same way. Only occasionally liver sympathin also caused a slight relaxation of the nonpregnant uterus.

Gaddum and Goodwin (50) gave particular attention to the question of whether the differences between liver sympathin and an injection of adrenaline might not be due to the difference in rate by which the active substances reach effective concentrations in the blood stream. Although in their experiments a slow injection of adrenaline often produced an effect more reminiscent of that of liver sympathin than a quick injection, they were not able to reproduce the effects of liver sympathin in this way; they concluded that their observations were more easily explained by assuming that liver sympathin was not adrenaline. They found that liver sympathin resembled in its effects that of a slow injection of 1 mg./minute of tyramine or of 10 µg./minute of noradrenaline.

It must be noted that Cannon's views on the nature of sympathin E were chiefly based on observations on liver sympathin; stimulation of other sympathetic nerves always produced responses more reminiscent of those to adrenaline. Liver sympathin has never been removed from the blood stream and tested outside the animal in which it was produced. Studies of this kind have been made on sympathin derived from other sources. Bacq (2) removed the aqueous humour from rabbits and dogs after sympathetic stimulation and found that the active substance was adrenalinelike in its pharmacological effects, color reactions, and ultraviolet spectrum, but no attempt was made at the time to differentiate between adrenaline and noradrenaline. This was done in experiments on the perfused rabbit ear by Gaddum and his colleagues (51,53,54). They found that after sympathetic stimulation the perfusate contained a substance pharmaco logically indistinguishable from adrenaline; it also gave the specific color reaction in the arsenomolybdate test (124).

In the earlier work, differences between adrenaline and sympathin were well-established only for liver sympathin, but the evidence for sympathin from other sources was less convincing. It is true that Cannon and Rosenblueth (29) had seen quantitative differences between adrenaline and the material released after the stimulation of the cardio-accelerator nerves, but the differences were not very marked. Progress followed upon the development of new methods for the identification of sympathomimetic amines in blood (54a). Using these methods, Peart (108a) was able to establish that the active substance present in the splenic vein of the cat after stimulation of the splenic nerves is mainly noradrenaline.

### B. ORIGIN OF SYMPATHIN

It is difficult to decide in each case if the active substance found after sympathetic stimulation is in fact released by the postganglionic neurons. The presence of chromaffin cells in the sympathetic ganglia and in the peripheral organs has already been discussed, and it is possible that these cells have been partly responsible for the effects ascribed to sympathin. There exists, however, experimental evidence which suggests that the postganglionic neuron or its endings are the source of sympathin. Bacq and Brouha (5) observed a small rise in frequency of the heart beat in a cat after the stimulation of the peripheral end of the sciatic nerve. Rosenblueth and Cannon (120) stimulated the lower abdominal sympathetic chains in the cat after severing most of its nervous connections, so that the erection of hairs over a small area of the skin of the rump was the only visible response. The stimulation caused also a contraction of the denervated nictitating membrane. After the removal of the area of the skin which had shown the response, stimulation was repeated, but there was no contraction of the nictitating membrane.

### C. ADRENALINELIKE SUBSTANCES RELEASED FROM ADRENERGIC AXONS

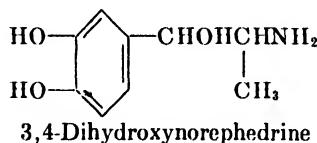
Gaddum and Khayyal (52) found that on electrical stimulation sympathetic fibers released a substance which had adrenalinelike actions on the frog heart. There is some doubt whether in these experiments the active substance was liberated only at the point of stimulation or all along the adrenergic fibers; this observation, however, indicates that the fibers do contain active material not only at the nerve endings. Similarly, Lissák has found that an adrenalinelike substance was released by the superior cervical ganglion after section of the preganglionic fibers or from the mesenteric plexus on electrical stimulation (91). In this work the contribution of the chromaffin cells of the sympathetic system does not seem to have been taken into account; it is not known whether they release adrenaline or a related substance under these conditions.

A recent contribution is that of Euler and Aström (45d) who have shown that active material is released when the isolated splenic nerves of cattle are electrically stimulated. The nerves were stimulated near one end, and the other end dipped into the fluid, which was assayed. Under suitable conditions an inhibition of the tone of the isolated intestine of the guinea pig was obtained when the bath contained fluid collected during the period of stimulation, indicating the presence of an adrenalinelike substance, but the substance was not fully characterized. In addition to this substance, histamine was released from the nerve on stimulation.

### D. SYMPATHOMIMETIC SUBSTANCES IN TISSUE EXTRACTS

Extracts from sympathetic ganglia and nerves, as well as extracts from organs innervated by adrenergic fibers, have adrenalinelike properties. Loewi's experiments on extracts of the frog heart have already been mentioned (93); von Euler (43) has recently confirmed Loewi's finding that the active material is L-adrenaline. According to Cannon and Lissák (28), the cat heart also contains active material; in two experiments the adrenaline equivalents were 0.5 and 0.8  $\mu\text{g}./\text{g}$ . heart, respectively. Hearts from sympathectomized cats gave inactive extracts. Extracts with adrenalinelike action have recently also been prepared by McDowall (97). An extract from cat hepatic vessels had adrenalinelike properties, but had no inhibitory action on the nonpregnant uterus of the cat (28). Active material was also found in extracts of sympathetic ganglia and fibers (90); the phrenic nerve and the cervical vagus did not contain an adrenalinelike substance.

More recently, tissue extracts have been studied by von Euler (41,42), who finds that the sympathomimetic substance extracted from the tissues behaves more like a primary amine than like adrenaline. Two primary amines were compared to the tissue extract in this work, 3,4-dihydroxynorephedrine and noradrenaline. Both these substances have in common an action on the arterial blood pressure of the cat which differs from that of adrenaline; their pressor action is not reversed by doses of ergotoxine which will produce a fall of blood pressure after adrenaline. The suggestion is made that the active material in the tissue extracts is in fact identical with L-noradrenaline. The only place where von Euler finds adrenaline is the frog heart (43).



In his first publication, von Euler described the properties of a pressor substance present in the spleen of cattle. This substance was contaminated in the crude acid-alcohol extracts by a depressor agent which could be removed by treatment with a lipide-ether mixture. The pressor substance was soluble in this mixture and could subsequently be eluted into an aqueous solution of sodium sulfate. The pressor action, expressed in terms of adrenaline, was 10  $\mu\text{g}./\text{g}$ . tissue; the assay was done on the cat, usually after a small dose of ergotamine tartrate (0.1 mg./kg.); this was given in order to increase the sensitivity of the preparation. Using a similar extraction technique, he found in extracts of mammalian hearts (from cattle,

horses, and cats) an adrenaline equivalent of 1  $\mu\text{g}./\text{g}$ . tissue. The pressor agents from both spleen and heart differed from adrenaline in the same way as noradrenaline; there was no reversal of the pressor effect after a large dose (2 mg./kg.) of either ergotoxine or ergotamine tartrate. In addition, the extracts showed little, if any, fluorescence in the Gaddum and Schild test.

von Euler's studies have recently been extended to nervous tissue. The activity of the tissue extracts in these experiments was assayed directly against noradrenaline, which had not been available in Stockholm during the war. The pressor effects of the extracts were compared to those of both DL-noradrenaline and L-adrenaline in the same preparation. The properties of the extracts were found to be similar to those of noradrenaline (43-45a).

TABLE II  
NORADRENALINE CONTENT OF NERVOUS TISSUE (45a)

Tissue	Activity of extracts, $\mu\text{g}.$ DL-noradrenaline hydrochloride/g.
Splenic nerves (freed from sheath).	10-30
Sympathetic chain	3-5
Cutaneous sensory nerves	0.5-1
Phrenic nerves	0.5
Ciliary nerves	0.8
Optic nerve	0.3
Anterior roots	0.5
Posterior roots	0.5
Spinal cord	0.2
Vagus	0.1

The present writer is grateful to Professor von Euler for the data reproduced in Table II, which gives noradrenaline equivalents in various parts of the peripheral nervous system. The figures differ slightly from those already published (45); in the present work the contributory effect of histamine present in some of the extract was eliminated by the use of an antihistamine drug. Also the extraction technique has been simplified.

Bacq and Fischer, using the method described earlier, confirm the occurrence of noradrenaline in extracts of the spleen of cattle, horse, and dog. In extracts of splenic nerves and of the sympathetic chain of the calf and the horse they find a mixture of adrenaline and noradrenaline. Extracts from human coronary arteries are said to contain only adrenaline (6).

A more detailed account of the nature and amounts of sympathomimetic material present in extracts of splenic nerves and of spleen in cattle is given

in a later paper by Euler (45c). The whole spleen contained 2-4  $\mu\text{g}$ . of L-noradrenaline per gram of tissue, the splenic nerves contained 10-15  $\mu\text{g}./\text{g}$ . The adrenaline content of the splenic nerves amounted to not more than 0.5  $\mu\text{g}./\text{g}$ . It is interesting that the spleen contained as much as one-fifth of the amount of noradrenaline found in the nerve; this suggests that the material is present in high concentration in or around the endings of the nerve fiber.

A careful study of the sympathomimetic activity of extracts from blood vessels of the horse and of cattle (121a) shows that the active material behaves like noradrenaline; this is true also for the extracts of coronary vessels of the horse. Only in one extract was the activity adrenalinelike: this was an extract from the renal artery of the horse.

The term "Sympathin" is used by Euler and Bacq for the sympathomimetic substance present in these tissue extracts. Their definition of the term differs from that given by Cannon, who believed that the transmitter was identical with L-adrenaline and only transformed to sympathin after its release by the nerve. Liver sympathin or any active sympathomimetic principle found in blood or perfusates after sympathetic stimulation are what used to be called sympathin; it seems doubtful if the term should be extended to the active material found in tissue extracts. Euler (45b) recommends the use of the terms "Sympathin N" or "Sympathin A." However, when the nature of the active agent has been established, there seems no reason why it should not be simply called adrenaline or noradrenaline.

#### E. SYMPATHOMIMETIC SUBSTANCES IN BLOOD

Earlier data on the adrenaline content of mammalian blood differ widely (for references see Jørgensen, 80). Some of the more recent figures have been compiled in Table III. There is too much difference between the data obtained by various authors to give one much confidence in the reliability of the determinations. There is reasonably good agreement between the data given by Jørgensen (80) and Kobro (83,84), but it is difficult to reconcile them with those of Bloor and Bullen (20); these authors find that the arsenomolybdate method reveals the presence in human and dog's blood of a substance other than adrenaline which is present in an approximate concentration of 0.25  $\mu\text{g}/\text{ml}$ . This is in agreement with findings by Raab (117). von Porat (116) finds that the fluorescence method, when used in plasma after removal of the proteins, will indicate the presence of as little as 0.25  $\mu\text{g}$ . adrenaline/ml., but no adrenaline was detected. Using the fluorescence method, Staub and Klingler (128) find that the method will detect 0.5  $\mu\text{g}/\text{ml}$ ., but no adrenaline was found in human plasma.

More recently, von Euler and Schmitterlöw have reported sympathomimetic activity in preparations from whole blood, human and bovine; the activity resembled that of DL-noradrenaline rather than that of adrenaline (46). This finding seems to open up the question of the nature of the adrenalinelike agent afresh.

TABLE III  
ADRENALINE OR NORADRENALINE CONTENT OF RABBIT AND HUMAN BLOOD

Species	Adrenaline (or noradrenaline) content, $\mu\text{g./ml.}$	Method <sup>a</sup>	Substance identified as <sup>b</sup>	Ref.
Rabbit . . . . .	0.074	Fl	—	80
Rabbit..... . . .	0.107	As	—	84
Rabbit..... . . .	0.098	Biol, As, Fl	A	142
Man . . . . .	0.068	Fl	—	80
Man... . . . .	0.044	As	—	83
Man . . . . .	<0.001	As	—	20
Man..... . . . .	0.02-0.04	Biol	NA	46

<sup>a</sup> Fl = fluorescence method; As = Arsenomolybdate method; Biol = Biological method (blood pressure, frog heart).

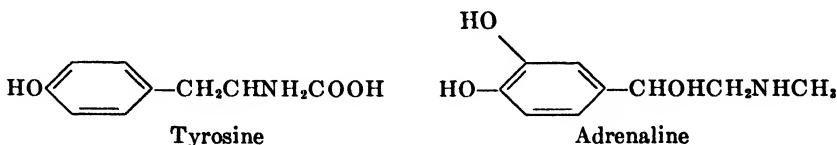
<sup>b</sup> A = adrenaline; NA = noradrenaline.

#### IV. Biosynthesis of Adrenaline

It is not known how adrenaline is formed or where it is formed. Both questions are interrelated. It is obvious that the adrenaline molecule must have been elaborated from dietary constituents which we will call the "primary precursors" of adrenaline. These primary precursors differ from adrenaline in more than one detail of chemical structure. Therefore, several steps are involved in the synthesis of adrenaline, and it is quite possible that these steps do not all occur in the same place, e.g., the suprarenal medulla. We have to consider the possibility that one or more precursors of adrenaline circulate in the blood stream, possibly on their way to the suprarenal gland. Such precursors may or may not have sympathomimetic properties. The stages by which the adrenaline molecule is built up are therefore of pharmacological as well as of biochemical interest.

Tyrosine or phenylalanine are usually considered the primary precursors of adrenaline; of all known constituents of the diet their structure is most similar to that of adrenaline. The conversion of phenylalanine to adrenaline has been demonstrated by the use of radioactive tracer technique (65a); these experiments show that in the synthesis of adrenaline both the aro-

matic nucleus and the side chain are derived from phenylalanine. The conversion of phenylalanine to tyrosine has also been established (103a). The close relationship between tyrosine and adrenaline is obvious when their formulas are compared:



Inspection shows that the formulas differ in four points: (1) adrenaline contains an additional phenolic hydroxyl group, in meta position to the side chain, (2) adrenaline has a hydroxyl group attached to the  $\beta$ -carbon atom of the side chain, (3) adrenaline has no carboxyl group, and (4) adrenaline has a methyl group attached to the nitrogen atom. Thus the minimum of chemical reactions which have to occur in the formation of adrenaline from tyrosine is four. These are: two oxidation reactions, one decarboxylation, and one *N*-methylation. These are all reactions of a type familiar in mammalian metabolism, and it is therefore permissible to inquire if the synthesis of adrenaline does in fact occur along these lines.

Theoretically, a sequence of four reactions gives a possibility of  $4! = 24$  different pathways of adrenaline synthesis. In the following treatment of some of these reactions which have been studied, we shall discuss which of these 24 theoretically possible pathways are best supported by experimental evidence.

#### A. DECARBOXYLASES

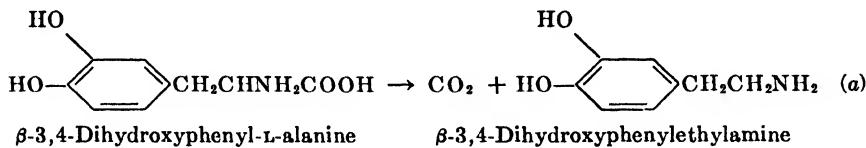
The decarboxylation reaction is of particular interest, as this is the step in which the pharmacologically inert amino acid is converted to a substance with sympathomimetic properties. The following is a summary of what is known about the decarboxylation of possible precursors of adrenaline and the enzymes which bring about these reactions.

##### 1. Tyrosine Decarboxylase

In earlier schemes of adrenaline biosynthesis the decarboxylation of tyrosine with the formation of tyramine was often considered an intermediate step, although the available experimental evidence was negative (48). It is not intended to give a full discussion of the older literature. There seems to be general agreement that a very feeble tyrosine decarboxylase exists in mammalian tissues (74), but the activity of this enzyme is very low and it cannot be studied by manometric measurement of the carbon dioxide formed in the reaction (for literature see 16).

## 2. Dopa Decarboxylase

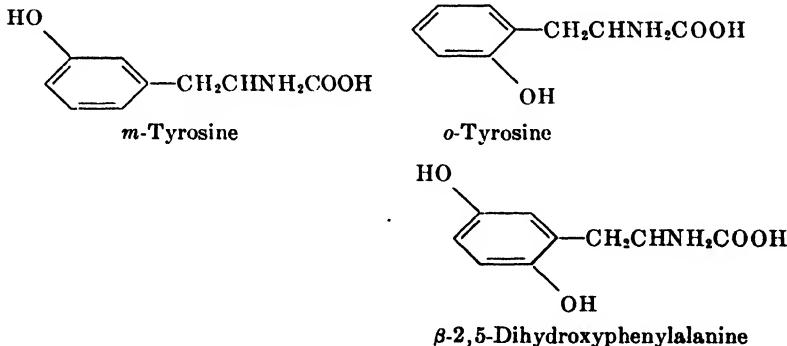
Interest shifted to this enzyme (dihydroxyphenylalanine decarboxylase), which was discovered by Holtz and his colleagues in 1938 (77). The enzyme will bring about the reaction:



This reaction represents a possible intermediate step in the synthesis of adrenaline (14).

Dopa decarboxylase was discovered in the mammalian kidney (77); it also occurs in the liver (14) and in the mucous membrane of guinea pig small intestine (76). The enzyme is not found in the suprarenal gland (18). Dopa decarboxylase is closely related to the bacterial L-amino acid decarboxylases (16,56). In liver extracts from rats reared on a diet deficient in pyridoxine the enzymatic activity is greatly reduced; the activity in such extracts can be partly restored by adding either pyridoxal and adenyl pyrophosphate (ATP) or synthetic codecarboxylase *in vitro* (17). As in the bacterial enzymes of this group, pyridoxal phosphate seems to be part of the complete enzyme system (see also 62).

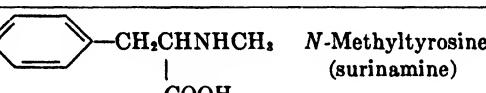
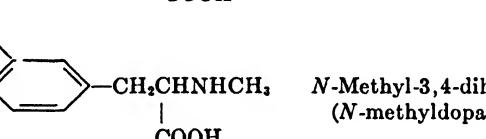
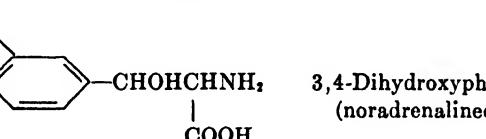
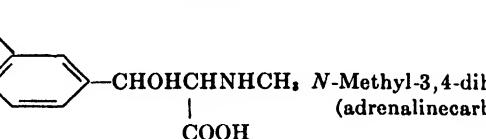
Three other amino acids have been found which are substrates of the enzyme; they are *m*-tyrosine, *o*-tyrosine, and  $\beta$ -2,5-dihydroxyphenyl-L-alanine (16a,19):



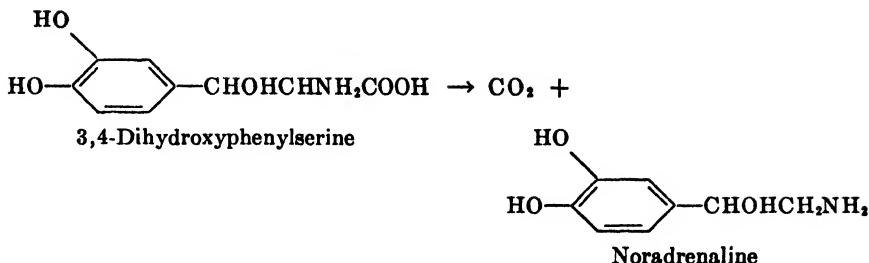
It is difficult to see how these two substances can be precursors of adrenaline and they will therefore not be discussed here. (A scheme of formation from phenylalanine via *m*-tyrosine cannot be excluded, but since *m*-tyrosine has never been found in the body, there is no experimental evidence in support of such a scheme.)

A number of other amino acids have been tested as possible substrates of dopa decarboxylase, which might qualify as possible precursors of adrenaline; these are listed in Table IV. Like tyrosine itself, they were found not to be substrates of the enzyme. (Amino acid (III) is very slowly decarboxylated by mammalian extracts). The substances were also tested with extracts of the guinea pig suprarenal gland and found not to be decarboxylated. It seems therefore unlikely that these acids act as precursors of adrenaline. This is of interest, as all the acids listed in the table would form sympathomimetic amines on decarboxylation. Amino acid (I) has been found in nature: its L form occurs in a number

TABLE IV  
POSSIBLE ADDITIONAL PRECURSORS OF ADRENALINE

(I)		N-Methyltyrosine (surinamine)
(II)		N-Methyl-3,4-dihydroxyphenylalanine (N-methyldopa)
(III)		3,4-Dihydroxyphenylserine (noradrenalinecarboxylic acid)
(IV)		N-Methyl-3,4-dihydroxyphenylserine (adrenalinecarboxylic acid)

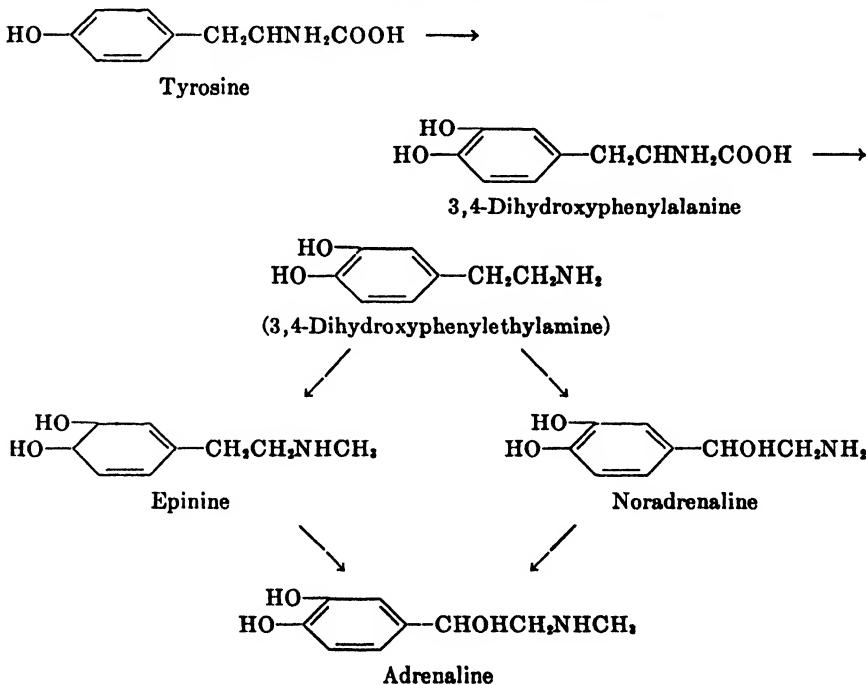
of leguminous plants, where it has been described as surinamine, ratanamine, or angeline (69). Amino acid (II) is a synthetic product (67); it does not act as a precursor of adrenaline in the perfused suprarenal gland of cattle (68). Amino acid (III) was first synthetized in 1919 (121); it has since been shown to be without action on the arterial blood pressure and the blood sugar level of the rabbit after a subcutaneous injection of as much as 100 mg./kg. (65). The acid has recently been prepared again (33); this specimen was decarboxylated by acetone-dried preparations of *Streptococcus faecalis R* (18). Such preparations are known to contain a very active decarboxylase for L-tyrosine (40,134). The reaction catalyzed is:



It was shown that the amine formed by the bacterial enzyme was the levorotatory stereoisomer of noradrenaline. The closely related *N*-methyl-amino acid (IV), recently prepared by Dalglish and Mann (33), is not decarboxylated by the mammalian or the bacterial enzymes.

SCHEME I

## PATHWAYS OF BIOSYNTHESIS OF ADRENALINE



Thus, of all the likely amino acid precursors of adrenaline, 3,4-dihydroxyphenylalanine is unique in being decarboxylated in tissue extracts at a reasonable rate; this gives support to the suggestion that reaction (a) is an intermediate step in adrenaline formation. The reaction takes place

not only *in vitro*, but also in the intact organism. Holtz has found a pressor substance with the characteristic properties of 3,4-dihydroxyphenylethylamine in the urine of rabbits and human subjects after oral or subcutaneous administration of 3,4-dihydroxyphenylalanine (75); this has been confirmed by other observers (73).

Let us therefore consider reaction (a) as an intermediate step in adrenaline biosynthesis. It can easily be seen that this would enable us to reduce the number of theoretically possible pathways from 24 to 2. These two pathways are shown in the Scheme I. This scheme differs from other schemes of adrenaline synthesis: it is based on a chemical reaction actually shown to occur in the organism. This fact alone is not sufficient to consider it as established, but it seems worth while to examine its implications.

### B. PHENOLOXIDASE

The scheme would require that the introduction of the second phenolic hydroxyl group is the first step in adrenaline synthesis. The formation of dopa from tyrosine is a reaction which has often been studied in invertebrates and in plants, where it is catalyzed by copper-containing enzymes, known as tyrosinases. In vertebrates, however, it is less easy to obtain evidence of the formation of 3,4-dihydroxyphenylalanine from tyrosine; here the main pathway of tyrosine degradation probably goes via a 2,5-dihydroxybenzene derivative, homogentisic acid (2,5-dihydroxyphenylacetic acid) (106, 107). The tyrosinase type of oxidation represents only a secondary pathway in vertebrates, e.g., in the formation of adrenaline, possibly also in the formation of melanin. A tyrosinase has been found in extracts from melanotic tumors of the skin of mice (72). Medes (98) reports the occurrence of 3,4-dihydroxyphenylalanine in the urine in tyrosinosis, a rare disorder of tyrosine metabolism in man.

### C. N-METHYLATION

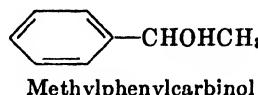
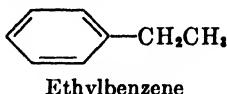
Nothing is known about the origin of the *N*-methyl group in adrenaline, but it seems probable that the formation of adrenaline, like that of other substances which contain labile methyl groups, depends on the presence of methyl donors. After liver and kidney, the suprarenal gland is most active among all organs examined in storing radioactive material after the feeding of methionine with a C<sup>14</sup>-labelled *S*-methyl group (94c). Choline is known to occur in the gland in high concentration (49,78). A study of the methylation of noradrenaline in the homogenate of suprarenal glands (dog and cat) has recently been made by Bülbring (25a); in this work the first direct evidence of adrenaline formation from noradrenaline was obtained. In these experiments, the addition of adenosine triphosphate

(ATP) to the homogenates was found to be essential for conversion, but it remains to be determined if the addition of choline was also essential.

There is evidence also that in the perfused dog's suprarenal gland added noradrenaline is converted to adrenaline (26b).

#### D. INTRODUCTION OF THE SIDE CHAIN HYDROXYL GROUP

Scheme I assumes that this reaction takes place after decarboxylation. There is no experimental evidence at present to show that this reaction occurs in chromaffin tissue or elsewhere. The conversion of ethylbenzene to methylphenylcarbinol:



in the rabbit is an example of a reaction of this kind (105,145).

#### E. SYNTHETIC ABILITIES OF THE SUPRARENAL GLAND

Earlier attempts to study the formation of adrenaline from added precursors in tissue slices (23,36,122, 135a) suffer from the fact that the adrenaline assay was mostly done by colorimetric methods of low specificity. The chief interest of Devine's experiments lies in the observation of increases in "adrenaline" content on incubation (36). This indicates that the gland did in fact produce adrenaline during incubation. On the other hand, changes of the adrenaline content depend on the rate of disappearance of adrenaline as well as on the rate of formation; such experiments are therefore difficult to interpret.

#### F. OCCURRENCE OF NORADRENALINE IN CHROMAFFIN TISSUE

The possibility that epinine is a precursor of adrenaline (see Scheme I) has been discussed (34a,77a). This cannot be excluded; there is, however, no evidence to support it. On the other hand, the role of noradrenaline as "Sympathin E" or "Sympathin N," and its presence in extracts of sympathetic nerves and other tissues, led to a search for noradrenaline in chromaffin tissue. There is now ample evidence to show that noradrenaline is a normal constituent of the suprarenal gland. Since the first publication by Holtz and by Schümann (77b), this has been confirmed in a number of other laboratories (26b,45e,61a). Not only is noradrenaline present in the gland, but it is now also established that it is released from the gland, *e.g.*, on stimulation of the splanchnic nerves (26a, see also 77b,98a).

An important practical outcome of these findings is the fact that commercial preparations of "epinephrine," *i.e.*, of the hormone extracted from

the gland, differ from synthetic adrenaline. The former contain usually about 10–20% noradrenaline (1a,133a).

The discovery of noradrenaline in human pheochromocytoma has already been mentioned; these tumors usually contain a much higher percentage of noradrenaline (61a,73a).

### V. Relation between Adrenaline and Sympathin

Biochemical data on adrenaline synthesis are far from complete. A few facts, however, emerge which are of interest in connection with the question of sympathin. We have seen that there is no evidence for the occurrence of decarboxylation in the suprarenal gland. This suggests that in adrenaline synthesis the origin of the sympathomimetic activity is outside the suprarenal gland. Amine formation appears to be mainly a function of the liver and the kidneys.

These considerations lead to the suggestion that liver sympathin is a precursor of adrenaline. The release of liver sympathin may be a function not only of the adrenergic nerve endings, but also of the liver cells. Sympathetic stimulation may act by causing a release of noradrenaline which has ultimately to reach the sites of adrenaline formation in the chromaffin tissue, possibly also in some adrenergic neurons. Such a concept would explain the findings of Euler and Schmitterlöw that noradrenaline normally occurs in blood (46).

Both Euler and Bacq are inclined to identify the substance found in tissue extracts—which they characterize as noradrenaline—with the sympathetic transmitter. Possibly there exist nerve endings where adrenaline is the transmitter and noradrenaline the precursor, which is *N*-methylated when released; a suggestion of this kind has been made by West (142). This might account for the apparently contradictory statements that sympathin is identical with adrenaline and that it is identical with noradrenaline.

Biochemical evidence suggests that noradrenaline preceded adrenaline (this does not exclude the possibility that a demethylation of adrenaline might also occur). Whether this sequence is also true in phylogenesis is not yet known, but it appears likely. The exact nature of the chromaffin material in invertebrates, *e.g.*, in the earthworm, might throw some light on this question. Neither is it yet known if, in ontogenesis, noradrenaline precedes adrenaline. The high concentration of noradrenaline in chromaffin tumors of the suprarenal glands has already been discussed; it has not been determined if active material is also found in the so-called sympathogoniomata, tumors of a more immature cell type that does not give the chromaffin reaction.

Information as to the sites of formation of noradrenaline is incomplete.

If we accept hydroxytyramine as the immediate precursor, there should be an enzyme which oxidizes hydroxytyramine, but the existence of this enzyme has not yet been demonstrated. It may be located in the liver, or in the chromaffin tissue, or in sympathetic nerves or their endings.

The chromaffin cell seems to surpass the adrenergic neuron in ability to store the active material. This may explain why, at least in vertebrates, the adrenergic neurones do not give the chromaffin reaction.

Possibly the whole neuron contains the transmitter and the losses of transmitter at the endings are made good by a movement of active material along the axon.

An alternative suggestion has been made by Burn (27), namely, that the adrenergic nerve endings pick up adrenaline from the blood stream; this is based on the observation that in the isolated perfused limb the response to sympathetic stimulation decreases almost to vanishing point, but reappears on adding adrenaline to the perfusion fluid.

It is not intended here to give a full review of the mode of action of adrenaline. One aspect has recently been discussed by Burn, namely, the relation between adrenaline and acetylcholine (27b). According to Burn, the view generally taken that adrenaline antagonizes the actions of acetylcholine does not fully account for all the facts known. The view is expressed that adrenaline modifies rather than antagonizes the effects of acetylcholine.

The fate of adrenaline has been the subject of comprehensive reviews, which also deal with the inactivation of sympathomimetic amines (4a,10, 11,60); the reader is referred to these articles.

## VI. Newly Discovered Functions of Adrenaline

### A. RELEASE OF ADRENOCORTICAL HORMONE

In her studies on the output of cortical hormone by the suprarenal gland in dogs and in cats, Vogt (136) noted that the stimulation of the splanchnic nerve led to an increased secretion of cortical hormone. Intravenous infusion of adrenaline also caused a prolonged increase in cortical activity. These observations were followed up by the study of the effect of repeated injections of adrenaline in young rats (137). It was found that the lipide content of the suprarenal glands was increased, and, when the treatment was continued for 24 days, the weight of the glands was slightly, but significantly, higher. Injection of insulin in rats caused a depletion of the suprarenal cortex in lipides; this depletion was in some cases abolished after adrenal denervation; the insulin effect is therefore interpreted as due in part to release of adrenaline (138).

### B. ADRENALINE-INDUCED RELEASE OF HISTAMINE

In a series of papers Staub (125-127) has described an effect of intra-venous infusion of adrenaline in man, namely, an increase in plasma histamine, as determined by Code's method (31). Similar effects had previously been noted in cats (37). In Staub's experiments small amounts of adrenaline—0.2 to 0.3 mg.—were slowly injected, in a period of about 10 minutes. It was shown that in most subjects this caused a rise of the plasma histamine to about two to three times the initial figures. The maximum values of plasma histamine were found toward the end of the injection period; the increase of the pulse rate due to adrenaline had a maximum in the first few minutes of the adrenaline infusion, and it had subsided by the time the peak in the plasma histamine had been reached. Staub considers this adrenaline-induced release of histamine a compensatory process; he suggests that the effect of histamine is to counteract the circulatory effects of adrenaline. Cases of collapse after administration of adrenaline are interpreted as due to the release of histamine; the absence of these symptoms of circulatory collapse after an injection of 3,4-dihydroxy-norephedrine (see Formula, Sect. III, D) is ascribed to the lack of histamine-releasing properties of this amine. Staub finds that the antihistaminic drug Antistin (*N*-phenyl-*N*-benzylaminomethylimidazoline) prevents the adrenaline-induced release of histamine. These results are of interest, as they suggest that some of the actions of adrenaline are not directly due to its own action on the excitable tissues, but to the histamine released. It will be necessary, in the light of these observations, to examine which of the actions ascribed to adrenaline are in fact effects of histamine.

## VII. New Synthetic Sympathomimetics

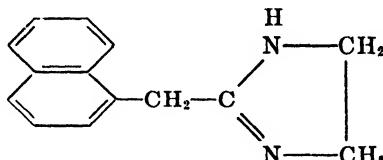
### A. BRONCHIODILATOR SUBSTANCES STRUCTURALLY RELATED TO ADRENALINE

In 1940, Konzett (87,88) studied a group of homologs of adrenaline in which the *N*-methyl group was replaced by an ethyl, isopropyl, propyl, isobutyl, or butyl group. Some of these substances were found to have strong bronchodilator action. The first two of these drugs were found to be more powerful bronchodilators than adrenaline. The isopropyl homolog was most active; it was about nine times as effective as adrenaline. In their action on the arterial blood pressure of the cat these substances had a depressor effect; this depressor effect was least marked with the ethyl homolog, where it was only apparent with small doses (2-30 µg./kg.); higher doses had a pressor action, which was enhanced after cocaine administration. In one experiment the pressor action of 300 µg. of the ethyl homolog was about the same as that of 15 µg. adrenaline. After

ergotamine administration, the ethyl homolog always caused a fall of blood pressure. All the higher homologs, including the isopropyl derivative, had a pure depressor effect.

### B. VASOCONSTRICTOR ACTION OF IMIDAZOLINE DERIVATIVES

The imidazoline derivatives include a number of substances of pharmacological interest. The first substance of this group examined is Priscol (2-benzylimidazoline) which has sympatholytic properties, *i.e.*, it is comparable in its actions to substances like ergotamine, yohimbine, or the substance 933F, mentioned in Section III, A (page 611); Priscol has chiefly vasodilator actions (100,104). The same group, however, includes a number of substances with a strong vasoconstrictor action (101). One of these is 2-(naphthyl-1'-methyl)-imidazoline, also known under the name Privine.



2-(Naphthyl-1'-methyl)-imidazoline

For a detailed description of the pharmacological properties of these drugs the reader is referred to the publications of Meier and his colleagues (99, 109); here we should only like to draw attention to the vasoconstrictor action. The group includes substances with stronger action than that of adrenaline. Moreover, their action is more prolonged than that of adrenaline. Pellmont and Meier have recently described the actions of the 4'-methoxy derivative of Privine; this substance produces about the same vasoconstriction as an adrenaline solution of tenfold strength; an even more powerful vasoconstrictor substance of this group (of as yet undisclosed chemical constitution) is also described (110). A study of the dose-effect curves of these drugs on the isolated perfused hind leg of the rabbit leads the authors to the conclusion that these imidazoline derivatives are true sympathomimetics with an affinity for the adrenergic receptors higher than that of adrenaline itself.

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CHAPTER IX  
Chemical Control of Nervous Activity  
**C. Neurohormones in Lower Vertebrates**

By GEORGE HOWARD PARKER

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**I. Historical Introduction**

Color changes in the lower vertebrates were apparently first described briefly though accurately in the chameleon by the Greek naturalist Aristotle (384-322 B.C.) and from his time onward these changes have excited the interest of students of the subject not only in the chameleon but in other reptiles, in the fishes, and in such lower vertebrates as the frogs and toads. These changes have been studied especially during the last century and it is now known that almost all cold-blooded vertebrates, fishes, amphibians, and reptiles exhibit them with more or less regularity.

It is further well established that such color alterations depend upon the integrity of the animal's eye and that, when the eyes are efficiently covered so that the experimental animal cannot see the tint of its surroundings, the changes whereby the creature may come to resemble its environment in tone no longer take place. This discovery led some of the older investigators of this subject to speculate on the significance of these color alterations. As such changes are always in the direction of adapting the color of the animal to that of its surroundings and thus to render it more or less lost to view, it seemed probable that they might be of advantage to the

creature exercising them in at least two possible ways: on the one hand, they might give it an advantage in the increased ease with which it might escape a possible foe by being unseen and thus avoid what might otherwise have been a disastrous collision, or, on the other hand, they might enable the creature to pounce unexpectedly upon prey which might serve it as food. Both these views are to be found in the speculations of the early workers and in fact are occasionally suggested as a possible reason for the origin of these changes. Thus color changes have been supposed by a number of the older investigators to serve in some instances as a protective and in others as a sustaining function in the habits of the animals possessing them.

## II. Chromatophores

Changes in tints in the lower vertebrates are produced by alterations in the color cells in the skins of these animals. The largest of such cells are visible to the unaided eye and are approximately the size of a period as used in the punctuation of this page. These color cells were first studied more than a century ago in the devil fishes, where they may be very large, by the Italian naturalist Sangiovanni, who called them "*cromofori*" from which their English name chromatophores is derived. In the lower vertebrates each chromatophore contains a mass of pigment which, spherical in form, may be located near the center of the cell, or the pigment as granules may be scattered through the whole chromatophore, in which case the cell is seen to be a body with numerous rootlike branches which together have a diameter several times that of the sphere of pigment. When the pigment in the color cells is contracted into a spherical mass, it has little influence on the tint of the animal as a whole and the color of the creature is pale. When, however, the pigment is fully scattered throughout the bodies of the color cells the animal takes on its most pronounced tint. All color changes lie between these two extremes and thus the color cells determine the momentary tint of any given creature.

In the lower vertebrates several kinds of chromatophores can be distinguished, as determined by the color of their contained pigment. The majority of chromatophores possess black pigment and are known in consequence as melanophores. Yellow pigment characterizes xanthophores and red, erythrophores; and white, leucophores. Each chromatophore as a rule contains pigment of only one color. When several kinds of chromatophores each with a distinguishing pigment enter into the formation of a more or less single body, as may not infrequently happen, the compound mass thus produced is known as a chromatosome. In chromatosomes the integrity of the individual chromatophores is probably fully maintained, notwithstanding the intimacy of the union of the several

cells. Different lower vertebrates may vary in the number of their chromatophoral pigments and consequently in the range of their color changes. The common catfish possesses only melanophores and has a limited color range, from a pale greenish-yellow to a coal-black. In the killifishes of the eastern U. S. coast in addition to melanophores there are xanthophores, leucophores, and green-blue chromatosomes. Hence these fishes change not only from dark to pale and the reverse, but they may also assume yellow, green, blue, or even red tints. The red coloration is due to an enlargement of the integumentary blood vessels and is, therefore, a true blush. The color changes in tropical lower vertebrates are like those in the killifish, except that they are much more exaggerated. In some of these creatures even the general color pattern seems open to alteration, almost to obliteration, as in the Nassau groupers. The southern flatfish, *Paralichthys*, will respond in experimental tests roughly to different-sized checkerboard patterns by becoming more finely or more coarsely spotted in accordance with the plan of checkerboard on which it rests. Such pattern changes, mostly due to melanophores, when coupled with true color changes, give tropical fishes an almost limitless range of tints.

The times required for these color changes are different in different lower vertebrates. The squirrel fish from Bermuda can change from a pale pink to a full red or the reverse in a few seconds while the New England catfish will require from one to several days to change from a pale gray to a coal-black. Frogs and toads are still slower in altering their body tints.

### III. Chromatophoral Nerves and Hormones

The all-importance of the eyes in color responses pointed at an early date to a probable nervous control of these activities, a view which gained more or less experimental support as time went on (Lister, 1858; Pouchet, 1876; von Frisch, 1910) until the early decades of the present century when this view became very generally accepted. That this interpretation was, however, not wholly consistent with all the observations that had been made even by some of the early experimentalists was evident. Thus in 1898 Corona and Moroni discovered that adrenaline when injected into a frog would induce profound color alteration. This unique observation was subsequently confirmed a few years later by Lieben, who made a more extended investigation of the subject. Comments by Fuchs on these two pieces of work led Redfield in 1918 to study the effects of adrenaline on the chromatophores in the horned "toad," *Phrynosoma*, with the result that adrenaline was found to be a potent means of inducing chromatic alterations. This led Redfield to the suspicion that possibly such internal secretions might play a considerable part in some animal color changes. Incidentally it was discovered that larval amphibians from which the

embryonic rudiments of the pituitary gland had been removed always developed into *pale* individuals. The significance of this condition in relation to the melanophore system was first clearly indicated by Atwell in 1919. Two years later Swingle observed that tadpoles into which a piece of pituitary gland had been transplanted became dark, and Hogben and his collaborators, especially Winton, were thereby led to an extensive and thorough investigation of this gland and its secretion in relation to the chromatic changes in lower vertebrates, with results that were truly revolutionary. The following summary presents the essentials of these studies.

The complete removal of the pituitary gland from a number of amphibians always left these animals in a permanently *pale* condition even though they were kept in an environment that, under normal circumstances, would induce them to turn *dark*. An examination of the skin of these pale individuals showed that the melanophores were in maximum contraction. The injection of pituitary extract into such animals was followed by a darkening due to the expansion of the melanophores, but this darkening was temporary, for sooner or later such individuals regained their original pallor. These results, when coupled with the negative outcome of experimental nerve cutting, led Hogben to conclude that in amphibians the color changes do not involve direct innervation, but depend upon fluctuating amounts of pituitary secretion. The possibility of a *dual* endocrine control was also suggested in 1931 by Hogben and Slome in their discussion of the conditions presented by the South African clawed toad *Xenopus*.

Frogs, like fishes, become pallid in a light-colored environment and dark in a dark-colored one, and lose much of this capacity when they are blinded. These facts suggest that the initial steps of the color changes in amphibians take place in their photoreceptors and are nervous. But, as the earlier part of this account shows, these nervous operations must excite secretory processes and the substances thus produced and poured into the blood become the effective elements in calling forth the color change. To this conclusion Hogben added the further important statement that if in these animals there is a nervous mechanism for regulating color control it is certainly a very subordinate one. In this respect amphibians seem to be the reverse of fishes in that the direct nerve control of the fish chromatophore is largely replaced by a hormonal one in the amphibian.

Are the melanophores in reptiles under the direct control of nerves as in fishes or under a hormonal control as in amphibians? In 1918 Redfield reported work in the melanophore system of the horned "toad" *Phrynosoma*. On stimulating the mouth of one of these lizards for some 5 minutes with a weak faradic current all the integumentary melanophores in this animal contract and the creature in consequence became pale. The spinal cord was then transected by Redfield at the thirteenth vertebra. On repeating

the oral stimulation the melanophores, previously expanded, contracted all over the animal's body as in the previous test. The lizard was again etherized and the adrenal glands removed. On stimulating the mouth once more the expanded melanophores contracted and the lizard became pale from the head back to the region of the cut but the melanophores posterior to the cut remained fully expanded. Redfield concluded from this and other like experiments that the melanophores of *Phrynosoma* are under a double control: a hormonal one, dependent upon adrenaline or some other like substance, and a direct nervous control. In their work on the African chameleon, Hogben and Mirvisch in 1928 conclusively showed that the melanophores in this lizard were under nerve control, but that, contrary to what had been demonstrated by Redfield in *Phrynosoma*, there was no evidence in this lizard of a hormonal influence. Both these pieces of work showed satisfactory evidence, for these two reptiles, of direct nervous control of melanophores similar to that already established for fishes, and in one of these there was also evidence of a supplemental hormonal influence.

Additional confirmatory evidence in favor of the direct nerve control of the melanophores in reptiles has been advanced by May for the southern lizard *Anolis*. One is, therefore, led to conclude that the chromatophore system in reptiles is much more like that in fishes than it is like that in amphibians. In reptiles it is essentially a nerve-controlled system exhibiting relatively little hormonal activity. When all these conditions in the color changes of the lower vertebrates are surveyed it must be confessed that these animals present strange contrasts. It is generally believed that the melanophore system in the lower vertebrates took origin in the fishes, was transmitted from them to the amphibians, and thence to the reptiles. That this system should have been first controlled in fishes directly by nerves, then in amphibians by hormones, and finally in reptiles again by nerves involves a succession of changes that must arouse scepticism in the mind of any zoologist.

#### IV. Neurohormones

##### A. TIMES FOR COLOR CHANGES

Of the color changes in the lower vertebrates and their relation to neurohormones none have been so extensively studied as those of the bony fishes, or teleosts. Some bony fishes accomplish these changes with extreme rapidity especially when the changes depend upon the red cells or erythrophores. *Holocentrus*, a red fish, was said by Smith and Smith in 1935 to change from red to white in some 5 seconds and from white to red in about 10 seconds. Parker records the red to white change in this fish

at an average of once in 6.38 seconds, and from white to red in about 18.7 seconds ( $19^{\circ}\text{C}.$ ), thus confirming in the main the statements of Smith and Smith. These changes are astoundingly rapid as compared to those of hours or days noted in the slower species. In *Fundulus*, the true color changes require several days or even a week. According to the observations of Hill, Parkinson, and Solandt, made by means of a photoelectric cell, the period for blanching and that for darkening in this fish were found to be nearly equal. Cole and Schaeffer, who also worked on *Fundulus*, state that blanching occurs in this species in a shorter time than darkening. Blanching, according to these workers, takes place 2.8 times more rapidly than darkening. The occasion of these discrepancies is not known.

Color changes in other fishes are relatively slow. Thus *Ameiurus*, according to Parker, may require as much as 36 hours to blanch and 19 hours to darken, periods that on repetition of the responses are soon reduced to approximately 3 hours and 1 hour, respectively. These observations have been substantiated by Matsushita on the oriental catfish *Parasilurus*. *Phoxinus*, according to Healey, changes from dark to pale in some 5 to 6 days and from pale to dark in from 1 to 2 days. These periods agree fairly well with the times recorded by Osborn for the two flatfishes, *Paralichthys* and *Pseudopleuronectes*, studied by him. These intervals of days are in strong contrast with those of seconds shown by such a fish as *Mollienisia*. Considering this striking range of times, it is not surprising that Hogben should have felt called upon to direct the attention of investigators to this aspect of the color problem.

The most lengthy interval ascribed to these changes for any fishes is that of about 20 days recorded by Neill in 1940 for both the darkening and the blanching of the European eel. These truly remarkable records stand in strong contrast to all others. As the following discussion will show, they may not be in reality fully comparable with the other shorter times. As reported by Neill, they were taken in water at a temperature of approximately  $8^{\circ}\text{C}$ . It is well known that, at this degree of cold, melanophore activity falls off very considerably. Wykes states that at  $6^{\circ}\text{C}$ . the color activities of *Ameiurus* are in almost full abeyance. It is, therefore, probable that the long time intervals for the color changes in the eel as reported by Neill may have been due in part at least to the temperature at which his work was done. At  $8^{\circ}\text{C}$ . the responses of melanophores must be greatly slowed down as compared to what they are at  $18^{\circ}$  to  $20^{\circ}\text{C}$ .—the temperatures at which many other records have been made. It must also be confessed that when Neill's plottings of these changes in the eel are inspected one is tempted to surmise that he has in some way failed to distinguish between what students of this subject have been accustomed to call physiological and morphological color changes. This surmise is

aroused by the fact that each of Neill's curves illustrating this part of his work is made up of a short interval of rapid change (about 10 hours), which may well be the physiological change, followed by a very long one of slow change (some 20 days), the morphological change. Such a separation in Neill's plots would bring his results more in harmony with what was originally given for the eel by Waring and by Waring and Landgrebe, whose records cover what would ordinarily be regarded as the period of color change. However, the difference in this respect between the work of Neill and of the two later investigators leaves the whole problem more or less obscured notwithstanding the qualifying comments recently made by Waring.

#### B. CAUDAL BANDS

When chromatic nerves are cut, particularly in the tails or other fins of fishes, temporary colored bands are produced that were seen in a number of lower vertebrates by the early workers. They were observed in several reptiles almost a century ago as the result of simple skin incisions. As early as 1852 Brücke recorded them in the skin of the chameleon and noted that the denervated areas were dark as compared to the rest of the skin. This is likewise true of fishes, as was shown by Pouchet in the turbots and Fukui in the filefishes. von Frisch employed this technique in tracing the course of the autonomic nerve fibers concerned with the color changes in fishes. In from half a minute to a minute after the autonomic tracts have been cut, the area thus denervated begins to darken. In 3 minutes, the dark area is very obvious and in 5 to 10 minutes it has reached a maximum deep tint. After 8 or 9 hours, it may begin to lose its depth of color. In *Phoxinus*, according to von Frisch, denervated areas at first fully dark begin to lose in tint after 8 days and finally at 13 days they may be as pale as that of the rest of the fish. The final blanching of these darkened denervated areas which appears to have been first recognized by von Frisch was a source of concern to him for he was unable to explain it.

Caudal bands are denervated areas of this kind formed in the tails of teleosts by cutting a bundle of radial nerves in this organ and thereby producing an elongated darkened area that will reach from the cut to the free edge of the fin. Such bands are extremely convenient in the study of the relation of nerves to chromatophores for they enable the investigator to compare denervated and innervated color cells at close range, in fact one next the other. Preparations of this kind were used by Wyman in his study of the relation of blood and nerve as chromatophore excitants. When care is exercised in selecting the position of the initiating cut, a band may be formed without interfering in any serious way with the blood supply to the denervated region. The relation of a successful cut to the blood vessels of

the tail has been clearly shown in a diagram by Fries, who employed caudal bands in his study of the xanthophores in *Fundulus*.

When an appropriately placed transverse cut about 1 mm. in length is made near the base of the tail in a pale *Fundulus*, a dark band will begin to appear in about half a minute and will grow in intensity of tint for some 5 minutes after which it will maintain itself for some time. In 6 hours or so the band will begin to fade and in about 2 to 3 days it will have become indistinguishable in the general pale field of the rest of the tail. If at this stage the fish is placed on a black background, it will darken except over the area of the caudal band which will stand out as a pale stripe in an otherwise dark tail. If such a fish is kept on a dark background for a day or so, the pale band first easily discernable will darken and be lost to view. If a caudal band is induced in a dark *Fundulus*, it will not be visible as long as the fish remains fully dark. If at any time the fish is made to blanch from having been put on a white background, the caudal band as a dark area will immediately stand out conspicuously to disappear in course of time by blanching gradually. Thus, caudal bands after their initial formation may be said to follow the tint of the fish on which they are formed, but with some considerable lag in time.

### C. INNERVATION OF CHROMATOPHORES

In the study of vertebrate chromatophores, it has been assumed from very early times that these color cells are under the control of the nervous system. So far as the teleosts are concerned, the work of two distinguished investigators, Pouchet and von Frisch, demonstrated by experimental means the correctness of this view. The foundations for this opinion were laid by Brücke in 1852; his studies were made not on fishes but on the chameleon. Brücke aptly compared the melanophoral nerve and its effectors, the color cells, to a nerve-muscle preparation. According to Brücke, when such a chromatic nerve acts, the melanophores contract as the skeletal muscles do under the influence of their motor nerves. When the chromatic nerve ceases to act, the color cells expand, which is the equivalent of the relaxation of skeletal muscle. From this standpoint, melanophore contraction would represent the active state of the color cell and expansion, the resting state. On stimulating melanophore nerves electrically, as was done especially by von Frisch, the color cells were seen to contract precisely as muscle cells do when their nerves are thus stimulated. On cutting a melanophore nerve, as is done in making a caudal band, the color cells expand just as a muscle relaxes when its motor nerve is cut. In the muscle, such a condition is due to paralysis and this interpretation of dark patches or bands produced by cutting chromatic nerves has been accepted by a host of investigators from the time of Brücke to the

present. Almost all the important workers of the past including Pouchet, von Frisch, and Sand have accepted this interpretation. This view of the nature of the caudal band and other like conditions has been designated by Parker as the paralysis hypothesis.

The chief change that Brücke's view has undergone of recent years came from Pouchet's discovery that the nerve fibers that control chromatophores are autonomic and not cerebrospinal as originally implied by Brücke. As cerebrospinal nerves control skeletal or cross-striated muscle and autonomic fibers innervate smooth muscle, it follows that the comparison of chromatophores with muscles is more truthful when the muscle chosen is not a cross-striated one but a smooth one. Smooth muscle, unlike skeletal muscle, is essentially a tonus muscle and chromatophores exhibit in their activities unusual degrees of tonicity; hence the appropriateness of a comparison of a chromatophore to a smooth-muscle cell. The details of this comparison so impressed Spaeth that he was led to declare in 1918 that a melanophore was a disguised type of smooth-muscle cell. Excepting this change from cross-striated muscle to smooth muscle, Brücke's original comparison of the neurochromatophore system in vertebrates to their neuromuscular system remains intact today.

The chief departure from Brücke's rather conservative scheme for chromatophore activation was that proposed by Zoond and Eyre in their study of the South African chameleon and reaffirmed by Zoond and Bokenham as well as by Sand in his general account of the color responses in reptiles and fishes. The view proposed by these workers was that, when a given chromatic fish or lizard is pale, it is so in consequence of a tonic contraction of its melanophores due to the action of autonomic pigmento-motor fibers. The paralysis that in the opinion of this group of investigators resulted when these fibers were cut was due, they believed, to the release of the melanophores from this tonus. On such a release these color cells would expand and thus the region of skin concerned would become dark. This expansion is believed to occur normally in animals in consequence of the inhibition of the ordinary tonic influences, an inhibition which could be called forth through either the direct illumination of the animal's dermal photoreceptors or the stimulation of its retina by light from a light-absorbing background. Stimulation of the retinal elements by light from a light-scattering background was believed to cause an inhibition of the original inhibition, thus allowing the tonic state to reassert itself. As a result the melanophore pigment would become concentrated and the animal would blanch. In commenting on this scheme, Sand was led to remark that it was by no means simple and that certain parts of it, such as the inhibition of an inhibition, were theoretically cumbersome.

A third view of the way in which nerves may control chromatophores,

counting Zoond and Eyre's departure from the older scheme of Brücke as a second one, is to the effect that chromatophores are supplied with two sets of nerves, one concerned with the concentration of pigment and the other with its dispersion. This view was first advanced by the French physiologist, Bert, in 1875 and has recently been revived by Parker. According to this view, the concentrating and the dispersing nerve fibers are antagonists and work one against the other. When for instance the eye of a fish is illuminated by light from a white background, the concentrating fibers are especially activated, the melanophores concentrate their pigment, and the fish blanches. When the eye is illuminated from a black surface, the dispersing fibers become active and the melanophores disperse their pigment, thus darkening the fish. Concentrating fibers are believed to be especially sensitive though not exclusively so to electric stimulation as compared with cutting. Dispersing fibers are more sensitive to cutting than to the electric stimulus. Thus cutting a bundle of melanophore nerve fibers excites the dispersing elements greatly and the concentrating ones at most very little, and hence a dark band is formed. Ordinary faradic stimulation, on the other hand, excites especially the concentrating fibers and hence produces a pale band or area. The two kinds of chromatophoral nerve fibers thus set off one against the other are supposed to act in opposition, as the sympathetic and parasympathetic fibers are believed to do, on the heart muscle of the higher vertebrates.

The three views thus described concerning the ways in which chromatophores and especially melanophores in teleosts may be controlled differ as to the kinds of nerve fibers involved and as to the way in which these fibers act. In attempting to reach a decision as to the validity of these views, it may be well at the outset to discuss the question of the kinds of chromatic nerve fibers in bony fishes. According to the first and second of these views, only one kind of nerve fiber is hypothesized. According to the third, two kinds should be present, one concentrating and the other dispersing.

That bony fishes possess concentrating chromatic nerve fibers is perhaps one of the most generally accepted views in color physiology. The evidence for this belief rests on the results of electric stimulation of nerves and nerve tracts as practiced by even the early investigators. When appropriate nerves, nerve tracts, or nerve centers are stimulated electrically, the part of the animal tested or, under certain conditions, the whole of the creature blanches with great regularity. If, previous to such a test, a part of the skin of the experimental animal is denervated, the blanching will in no way affect the part thus devoid of nerves. This general type of response has been recorded repeatedly in fishes and reptiles by numerous recent workers: in fishes by von Frisch, Spaeth, Schaeffer, Wyman, Parker,

Abramowitz, Osborn, Wykes, Parker and Rosenblueth, and Hunter and Wasserman, and in reptiles by Redfield, Hogben and Mirvisch, Sand, and Parker. Since in this type of blanching the color change never spreads into a denervated area, it cannot be due to a concentrating hormone carried in the blood but must result from nerves acting locally on the color cells. The evidence for this view is now so complete that no result in chromatic physiology is more generally accepted than the presence in many lower vertebrates, and particularly in the teleosts, of the concentrating type of nerve fiber (Sand).

Evidence for dispersing chromatic nerve fibers in bony fishes and other lower vertebrates has not been so easily obtained nor has that which has been discovered proved so generally convincing to workers in this field (Waring). Much of the evidence for dispersing fibers has come from

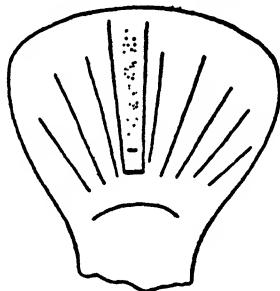


FIG. 1. Diagram of faded band in tail of *Fundulus* within which a new short cut has been made, with result that this cut induced formation of a new small band within limits of larger band (Parker, 1934).

work on caudal bands discussed in a preceding section of this report. The nature of caudal bands and other like areas so far as the present account is concerned can well be illustrated by what is to be seen in these bands in the killifish. If a caudal band is induced in the tail of a pale killifish, *Fundulus heteroclitus*, by cutting a bundle of radiating caudal nerves, and the fish is kept in a white, illuminated vessel, the band will fade in a few days. This kind of blanching in fishes was first pointed out by Pouchet. It was also noticed by von Frisch, but neither Pouchet nor von Frisch gave it any special consideration. If after such a caudal band has blanched a new transverse cut is made within the area of the old band and slightly distal to the original cut, a second band will appear reaching from the new cut over a part of the original band to the edge of the tail (Fig. 1). Such a revival of the greater part of the old band is a very significant and novel fact and leads to two important conclusions. The first is that that portion of the chromatic apparatus which has been severed from the central nervous system by the first cut is shown by the second cut to be

not paralyzed, as was claimed by Brücke, but capable of full, even of excessive, activity. And the second conclusion is that since the original cut must have obstructed completely all central influences such as tonus and the like, the revival of the band by the second cut must be due to a local stimulation of fibers in the severed nerve. Since under this kind of stimulation the melanophores of the band disperse their pigment, such fibers must be dispersing fibers. Hence the general conclusion has been reached from tests of this kind that the chromatic nerves of *Fundulus* contained dispersing as well as concentrating fibers, that is, the melanophores in this fish are dineuronic.

This interpretation of the work on the caudal bands of *Fundulus* has roused considerable opposition. However, since the attention of workers was called to the revival of faded caudal bands by recutting, this phenomenon has been recorded in a number of other fishes. It has been noted in the melanophore system of *Parasilurus* by Matsushita, of *Pterophyllum* by Tomita, and of *Ameiurus* by Parker, and in the erythrophore and xanthophore systems of *Holocentrus* by Parker, and of two species of *Fundulus* by Fries. The inability of Wykes, of Osborn, and of Vilter to revive by recutting the blanched bands in the teleosts studied by them appears to have been due either to the low temperatures at which they worked or the lateness when their tests were applied (Parker), for the fishes which these investigators studied unsuccessfully have since yielded positive results in the hands of other workers. The revival of caudal bands and other similar dark areas in teleosts by the recutting of their nerves may therefore be said to rest on a sound observational basis.

Although this revival of faded caudal bands in teleosts is now admitted by many workers, the view that such a revival necessarily implies the presence of dispersing nerve fibers has not proved generally acceptable. Several objections to this view have been raised by recent workers. It has been pointed out that, if caudal bands are due to the activity of dispersing fibers, this activity must continue as long as the band lasts, often several days, a period much too long for the continued activity of a nerve excited by a single cut. But it is by no means certain that the nerve is so excited. It is much more probable that its continued activity, like that of nerves of pain, is due to continuous stimulation from the cut itself. If this is true, a more or less regular flow of chromatic impulses must be passing over such nerves from the region where the impulses are believed to originate, i.e. the cut, to the color cells themselves.

This problem was attacked with a somewhat more incisive technique by Mills, who studied the melanophores on the edges of the caudal bands in *Fundulus*. After these bands are well formed, it is easy to cause the fish to change color by changing its backgrounds or stimulating the nerve tracts

electrically. When the edge of a caudal band is closely observed with the fish first pale and then dark, the limits under the two conditions do not coincide exactly. Moreover, on this edge melanophores will be found that can expand but not fully contract and others that can contract but not fully expand. It is very difficult to explain these conditions on the assumption of a single set of nerve fibers, but it is very easy to understand them if double innervation is admitted, for the margins of the band would be regions where through the accident of the cut certain melanophores would be deprived of concentrating fibers, but not of dispersing ones, and vice versa. It appears to be necessary to assume double innervation to understand these otherwise peculiar states.

Another line of evidence for double innervation was developed by Abramowitz and is concerned with the regeneration of the melanophore nerves in *Fundulus*. When these nerves are cut so as to produce a caudal band, the portion of the nerve distal to the cut degenerates completely in about two weeks, after which regeneration begins and progresses at a rate of approximately half a millimeter a day (Parker and Porter, and Abramowitz). When regeneration is well under way, the extent to which it has progressed can be seen on darkening a blanched fish by placing it on a black background. The caudal band will then darken as far as its nerve fibers have regenerated; the rest of the band will remain pale. If now the distal area of the regenerating band is photographed under the dark and the pale conditions of the fish and the individual melanophores in this area are studied critically, four kinds may be identified. Some melanophores will be found that are capable of full contraction, but not of expansion, others with full expansion but incomplete contraction, still others with full expansion and full contraction, and finally some responding neither by expansion nor by contraction. Those responses which go to completion in one direction but not in the other are difficult, if not impossible, to explain except on the assumption that the concentrated phase of the melanophore is dependent upon one set of nerve fibers and the dispersed phase upon another.

The evidence for the double innervation of the melanophore in *Fundulus* and in *Ameiurus* and for the erythrophores in *Holocentrus* as presented in the preceding paragraphs has been recently substantiated on other species of fishes by a number of important investigators in this field (Dalton and Goodrich, Tuge, Chang, Hsieh, and Lu, Healey, Tomita, Gelei, Fries, Lee). The insufficiency of this evidence seems to have impressed few but Waring. Since the recent work here referred to covers a considerable number of teleost species, it may well be that double innervation will prove to be the prevailing type of innervation among bony fishes. Whether the chromatophores of the chameleon are supplied with two sets of nerves, as was

originally maintained by Bert, is an open question and must await further investigation, but in the instances of the fishes already alluded to there appears to be no doubt that their chromatophores are dineuronic and that their caudal bands and other like dark areas are due to the excitation of dispersing nerve fibers and not to paralysis, the blocking of central tonic impulses or other like influences. When, however, experimental steps are taken to prevent the interference of the concentrating fibers, antidromic responses in the form of the expansion of melanophores proximal to the cut can be demonstrated readily not only in *Fundulus* (Parker), but also in *Ameiurus* (Parker). Such responses have also been identified by Tomita in the angelfish *Pterophyllum*. Although this type of response was sought for in the young of *Mustelus*, it was not found (Parker). Thus, at least in certain teleosts, dispersing melanophore nerve fibers, like ordinary motor fibers, exhibit under appropriate conditions antidromic activities.

#### D. INTERMEDIATE

The fact that teleosts possess both concentrating and dispersing chromatic nerve fibers does not preclude the occurrence in them of other means of color control such as hormones. Chief among these may well be intermedine. The term "intermedine" is here used to designate the chromatophore-activating principle or "B" hormone contained in the intermediate lobe of the vertebrate pituitary gland. A detailed account of the most recent knowledge of this hormone is given by Waring and Landgrebe in Chapter VIII.

The effect of this principle on teleost melanophores was variously described by the early workers. Spaeth, Wyman, and Gilson stated that the pituitary extract produced contraction of the melanophores in *Fundulus*. Abolin, who worked on *Phoxinus*, was unable to confirm this statement and reported that in the minnow both melanophores and xanthophores expanded when subjected to this agent. Abolin also noted an expansion of chromatophores when the pituitary extract was injected into *Nemachilus*, *Esox*, *Carassius*, and *Leuciscus*. Przibram, who worked on amphibians, attempted to explain away these differences on the basis of dosage. He declared that in frogs a low dosage resulted in a dispersion of the pigment and a high one in a concentration of color. This view was not substantiated by Abramowitz. It appears that attention must be given to the diversity of pituitary extracts, the production of which is far from uniform, and to possible differences in the chromatophores themselves even in the same fishes. Hewer found that the melanophores of *Phoxinus* contracted in response to an extract from the posterior lobe of the pituitary gland from the cod, and Meyer noted the same kind of reaction to pituitrin and hypophysin when used on *Gobius* and *Pleuronectes*. Matthews made the

interesting observation that a pituitary extract from *Fundulus* would contract the melanophores in an isolated scale from this fish though this agent had no effect upon the color of *Fundulus* when injected into the fish itself. Subsequent work showed that expansion of melanophores by pituitary extracts was the rule in *Ameiurus* (Odiorne, Parker, Abramowitz, Veil, and Osborn), in *Phoxinus*, *Gasterosteus*, *Rhodeus* (Osterhage), and in *Carassius* (Verne and Vilter). Xanthophores and erythrophores were also found to expand to such extracts. This was discovered to be true for *Gobius* and *Pleuronectes* by Meyer and for *Phoxinus* by Giersberg, by Peczenik, and by Lewis, Lee, and Astwood. The responses of *Fundulus* to pituitary extract and particularly to intermedine are somewhat peculiar. Desmond noted that the loss of the pituitary was without effect on the melanophore reactions of young *Fundulus*, although a transplant of the pituitary gland from this fish into hypophysectomized axolotls or tadpoles darkened these animals. Matthews also discovered that a *Fundulus* without a pituitary gland could change to dark or pale quite as a normal one did. Lee has very recently shown that pituitary secretion is not necessary for the color changes in the toadfish *Opsanus*. Matthews as already noted also observed that the melanophores in isolated scales from *Fundulus* contract when the scales are immersed in an aqueous extract of the pituitary gland from this fish. Kleinholtz made the interesting discovery that, when a *Fundulus* with a blanched caudal band was injected with pituitary extract from another *Fundulus*, the band darkened, showing that the melanophores of *Fundulus* when released from nervous control are open to the action of intermedine. Abramowitz was led to study from a quantitative standpoint the melanophore pituitary hormone in *Fundulus*. He found that the pituitary gland of a *Fundulus* 10-cm. long, when extracted with distilled water, yielded about 4 frog units of intermedine. Such a gland on chemical treatment could be brought to yield some hundred such units. About a fifth to a tenth of this amount will darken a pale caudal band in *Fundulus*. Blood from a single dark *Fundulus* when extracted and chemically treated also yielded enough intermedine to darken a pale band. Thus the blood of this fish carries in itself enough potential intermedine to be physiologically significant in color changes. Whether in the normal color responses of *Fundulus*, it is thus significant, as believed by Abramowitz, is still to be shown.

From these rather confused records covering the relations of pituitary extracts to chromatophores and particularly to melanophores, two conclusions seem to emerge: first, that the pituitary extracts used by many workers, particularly the earlier ones, were probably far from pure and doubtless contained accidental inclusions of no small significance in color changes; and, second, the black pigment cells of teleosts, though included

under the general name melanophore as though they formed a physiologically homogeneous group, are probably far from uniform in their reactions. The irresponsiveness of *Fundulus* melanophores, as compared to the reactivity of those in *Ameiurus*, to what appears to be a reasonably pure form of intermedine is indicative of a decided physiological difference between the dark pigment cells in these two fishes.

To the early workers on chromatophoral pituitary extract, it was soon evident that this activator could be obtained from the pituitary complex of any vertebrate from fishes to mammals (Hogben). This lack of specificity has been many times recorded by later investigators. Even extracts from what is regarded in tunicates as the homolog of the vertebrate pituitary complex have yielded not only an oxytocic principle but also a melanophore-expanding one as tested on frogs (Bacq and Florkin). As already noticed, the same general nonspecificity has been recorded again and again for the eye stalk extracts from crustaceans. Not only is there lack of specificity within phyla, but this also occurs between phyla. Thus Koller and Meyer and later Meyer showed that the crustacean eye stalk extract would activate the chromatophores of flatfishes, an observation that was soon extended to other chromatic vertebrates (Perkins and Kropp, Kropp and Perkins, and Abramowitz). Conversely vertebrate intermedine was shown to act upon crustacean chromatophores much as eye stalk extract does (Abramowitz, and Abramowitz and Abramowitz). In consequence of these reciprocal interphylar activities, a certain similarity, perhaps chemical in nature, may be assumed to exist between vertebrate intermedine and the crustacean eye stalk principle, and the chromatophoral reactions of animals, even as distantly related as those in these two groups, may be more intimate than their systematic positions would lead one to expect.

A method for making strong extracts of intermedine has recently been published by Landgrebe, Reid, and Waring (*cf.* Chapter VIII, Section IV, E). However, very little is known chemically about this substance. It is soluble in water and is carried in the blood and lymph from the pituitary gland, where it is formed, to the chromatophores where it acts. The blood of a dark fish when injected into a pale one will ordinarily induce the formation of a temporary dark spot, but such blood will not call forth a color change in a dark fish. Intermedine is a typical hydrohumor and must play an important part in the normal darkening of many teleosts. It is a reasonably stable material, for it can be identified in the blood of hypophysectomized fishes some 3 days after the removal of their pituitary glands (Osborn). Van Dyke describes it as: "the melanosome-dispersing or chromatophorotropic hormone of the pars intermedia. Probably this

hormone is a polypeptide. So far as we know the survival of cells secreting intermedine in the mammalian pituitary represents an atavistic heirloom since intermedine is principally of interest as a hormone dispersing pigment granules in cells of certain fishes, amphibia and reptiles." This is probably an inadequate statement, for the fact that intermedine is generously produced in almost all chordates from the tunicates to man is indicative of other functions than the purely chromatic one known in the cold-blooded vertebrates. What these functions may be is yet to be discovered. Waring and Landgrebe in Chapter VIII present evidence that the various metabolic effects ascribed to intermedine are in fact due to other principles. That intermedine has served to ameliorate the human ailment piebald skin or vitiligo is indicative of functional significance even in the human being. It is probably far from an atavistic heirloom.

It must be evident from what has been stated in this section and in the preceding one that some fishes, for instance the catfish *Ameiurus*, possess two means for darkening, dispersing nerve fibers and the pituitary gland. It may well be asked how these two mechanisms with the same function are related. When the pituitary gland is removed from a catfish, it has left only one means of darkening, the dispersing nerve fibers. Such fishes when put in a black, illuminated environment will darken only to about half the extent of a normal fish. In a fully pale catfish, the black pigment masses in the macromelanophores have a diameter of about  $45\mu$ ; in fully dark fishes these colored areas have diameters of some  $145\mu$ . In the hypophysectomized fishes, the areas of melanin have diameters of approximately  $100\mu$ . As already stated, the melanophores in this condition appear to be about half expanded. This is the maximum effect, in this fish, of activation by dispersing nerves. If into such a catfish thus brought to an intermediate tint some pituitary extract is injected, the fish will darken gradually to its full coloration, that is, the pigmented areas of its melanophores will change in diameter from about  $100$  to  $145\mu$ . The remarkable limitation to darkening by nerves, first noticed by Abramowitz and by Veil, brings out one striking difference between dispersing nerves and pituitary extracts as color cell activators.

Another difference between these two means of dispersing melanophore pigment in the catfish is seen in the rapidity with which the two agents act. If a pale catfish with a blanched caudal band is allowed to darken naturally in a black, illuminated aquarium, it will be seen in an hour or so to be wholly dark, except for the caudal band. Somewhat after an hour, this band will begin to darken until in a brief time the band is as dark as the rest of the tail. As the caudal band is denervated and does not darken in the beginning, the initial darkening of the rest of the animal must be due

to the dispersing nerves. Thus the deep tint must be initiated by the nerves. The darkening of the caudal band, which follows the general darkening of the tail, cannot be due to nerves, for the band is denervated. It must result from pituitary intermedine, and from now on the fish must continue to darken by the combined action of both dispersing nerves and intermedine. If this analysis is correct, it follows that of the two darkening agents in *Ameiurus* the color change must start through the action of the dispersing nerves to be followed an hour or so later by the intermedine from the pituitary gland. Which of these two agents maintains the dark condition of the fish, which under appropriate conditions may last indefinitely, cannot at present be stated. Very likely both are involved. What, however, is certain is that, in the darkening of *Ameiurus*, nerves are effective before the pituitary secretion comes into play, but are less complete in their action than is the pituitary agent (Parker).

#### E. ADRENALINE

The precision and uniformity of the responses of teleost chromatophores to adrenaline is in strong contrast with the uncertainty of their reactions to pituitary extracts. In 1916, Spaeth pointed out that the melanophores in the scales of *Fundulus* would contract to a solution of adrenaline (epinephrine) 1 part/10<sup>6</sup>. Subsequently, Barbour and Spaeth determined the limit of effectiveness of this solution to be 1 part/5 × 10<sup>7</sup>. They also pointed out that after a scale had been treated with ergotoxin, its melanophores would expand to adrenaline. The contracting action of this agent on melanophores was also observed on trout (Gianferrari) on *Fundulus* (Wyman, Abramowitz, and Bogdanovitch), on *Phoxinus* (Abolin, Giersberg, D. C. Smith, and Osterhage), on *Carassius* (Fukui, Beauvallet, Verne and Vilter), on flatfishes (Hewer, Meyer), and on *Ameiurus* (Bacq). As a blancing agent, adrenaline appears to be remarkably uniform and consistent in the color responses that it calls forth in teleosts. Bogdanovitch pointed out that this reagent was subject to destruction by the tissues of the scales in which the melanophores were naturally imbedded. Abolin added the interesting fact that in *Phoxinus*, though the melanophores were contracted by adrenaline, the erythrophores failed to react in any way to this agent.

Gilson, who confirmed Spaeth's original observation that adrenaline contracted the integumentary melanophores in *Fundulus*, showed that it caused a dispersion of melanin in the retinal pigment cells of this fish. Thus adrenaline when injected into a given fish would cause a contraction of its integumentary pigment and an expansion of its retinal melanin. These observations by Gilson confirmed what Fujita and Bigney had recorded and were in opposition to the statements of Klett. In Spaeth's

original test of the action of adrenaline on the melanophores in the scales of *Fundulus*, the scales as prepared by Spaeth carried with them live nerve endings as well as melanophores, so that it was impossible to decide which of these parts, the nerve or the color cell, was acted on by the reagent. Parker, therefore, prepared caudal bands in the tail of *Fundulus*, and after the nerves had fully degenerated and the fishes were darkened by being kept on a black background, he injected adrenaline into them. The innervated melanophores in general as well as the denervated ones in the caudal band contracted showing that this agent could act directly on such color cells. The same was found to be true of the melanophores of *Ameiurus*. Parker, therefore, sided with Lieben and Wyman in the belief that melanophores may be stimulated directly by adrenaline and opposed Fuchs, Spaeth and Barbour, and Abolin and Giersberg, who held that this agent excites the melanophores only indirectly through their nerves.

Veil found that the melanophores in the scales of the carp could be made to contract with adrenaline reduced to millionths in dilution. When this extreme efficiency of the adrenaline was set off against that of intermedine, it was estimated that the mildly active intermedine solution must be increased about 5000-fold to counteract the adrenaline. Veil had previously been led to conclude from a study of the melanophores in the scales of teleosts that the concentrating melanophore nerves in these fishes produced a substance at their terminals which was adrenalinelike in character.

As early as 1911 von Frisch noticed that dark denervated areas in *Phoxinus* would blanch quickly following excitation of the fish. This excitement pallor, as it has since been called, has commonly been looked upon as due to the sudden discharge into the blood in the fish of an amount of adrenaline sufficient to blanch the creature. Bray recorded that, when the catfish *Ameiurus* was raised to a high pitch of nervous excitement, it became blanched and remained so for a considerable time. Abramowitz also often noticed that catfishes paled when they were disturbed by attempts to capture them. Dark caudal bands in these animals would blanch under such circumstances and would remain in this condition several hours after the fish had again darkened. Similar effects were evoked by the electrical stimulation of the roof of the mouth, of the medulla oblongata, or the anterior end of the spinal cord. Some evidence was found by Abramowitz to the effect that the blood of such stimulated animals carried a substance that would blanch other dark individuals. Although this evidence is not finally conclusive, it points to the possibility of adrenaline or some like substance as a means of teleost blanching.

Adrenaline is a secondary alcohol which was synthesized over two decades ago. Its molecules are relatively small. It may well represent the

"W" substance of Hogben and be derived from such sources as the *pars tuberalis* of the pituitary complex (Hogben and Slome, and Hogben). It can act both as a hydrohumor and a lipohumor. Whether Cannon's sympathin is to be placed among the chromatic neurohumors of vertebrates, as Vilter, and Chung and Lu have done, is an open question. Adrenaline as a neurohumor is derived from two general sources, the medulla of the adrenal gland with other less-defined chromaffin masses and the concentrating chromatic nerve fibers. It is not impossible that the substance here described as adrenaline from the chromatic fibers is sympathin, but this question must be left for further investigation. A more detailed discussion of the relation between adrenaline and sympathin has been given by Blaschko in the preceding section of this chapter.

#### F. ACETYLCHOLINE

Acetylcholine, because of its well known importance as an activator of both neurons and effectors, has long been suspected of being an agent in the excitation of melanophores. However, with this agent as with intermedine the tests of the earlier workers gave little uniformity. Barbour and Spaeth observed no response to it by the melanophores of *Fundulus* even when it was used 1 part/1000. Negative results were also obtained by Wunder, who employed it on *Rhodeus*, and by Smith and Smith, who tested it on the melanophores of *Scorpaena*. Parker suspected it of producing a slight contraction in the melanophores of *Fundulus* and Beauvallet substantiated this view on the goldfish. Bogdanovitch declared in favor of contraction in *Fundulus*. Parker, who modified his technique by the preliminary use of eserine (physostigmine), found in a second set of tests on *Fundulus* evidence of a slight expansion rather than a contraction. Chin, who worked on the paradise fish, *Macropodus*, obtained a dark area on injecting acetylcholine into this creature and believed that he could identify this substance in extracts of the caudal fin of *Macropodus*. Chang, Hsieh, and Lu accepted these results and presented evidence to show that this agent is to be found in the chromatic nerve fibers of the snakefish *Ophiocephalus*.

It now appears after extended trials with this activator that it disperses melanophore pigment and thereby darkens fishes (Mendes). Acetylcholine when injected into a fish is rapidly destroyed by the cholinesterase of the animal's tissues. To prevent this destruction, it is necessary to prepare a chromatic vertebrate for the reception of acetylcholine by a preliminary injection of eserine. In the common catfish, *Ameiurus*, 1 part of eserine by weight may be injected for every 200,000 parts by weight of fish. In a series of catfishes thus prepared, each individual received 0.2 ml. of solutions of acetylcholine of different concentrations from 1 part of the

reagent in 100 parts of Ringer solution to 1 part/ $10^{11}$  parts solvent. The weakest of these mixtures had no effect on the color of the fishes. All others darkened them and the strongest two eventually brought on death. The satisfactory range of the solutions for physiological tests was from 1 part/ $10^4$  to 1 part/ $10^9$ . The darkening that resulted from these various concentrations usually required from 20 to 30 minutes to reach its maximum (Parker).

Acetylcholine is a choline derivative, the functional efficiency of which is enormously increased by acetylation. Its molecules are relatively small and readily inactivated by the cholinesterase of animal tissues. It is soluble both in water and in oils and can therefore act either as a hydrohumor or as a lipohumor. When dissolved in the lipides of the tissues, it is protected for a considerable period from destruction by cholinesterase (Parker).

#### G. SUMMARY OF NEUROHORMONES

Before 1910, and in fact for almost a decade after that date, all workers in this field were persuaded that the natural responses of active color cells were excited exclusively by nerves. These chromatic nerves, as already stated, were assumed to act upon the chromatophores as motor nerves do upon muscle. But after the work of Redfield on lizards and of Huxley and Hogben and of Hogben and Winton on amphibians it became increasingly evident that at least certain chromatophores were normally excited either exclusively or partly by hormones. In this way such agents as adrenaline, intermedine, and acetylcholine took their places as essential elements in the chromatic mechanism. For a time it seemed as though the activation of color cells as a whole was a mixed phenomenon, in some instances purely nervous, in others purely humoral, and in still others in part nervous and in part humoral. However, it soon appeared that nerves acted upon color cells not so much by virtue of changes in electric potentials, as had been held to be true by the older workers for the nervous system in general, but in consequence of substances discharged at the nerve terminals and in very close proximity to the color cells themselves. These substances induced in the chromatic cells a dispersion or a concentration of their pigment precisely as had been found for the neurohormones. In fact except for the closeness of their region of origin to the color cells, such substances were in no wise to be distinguished from true neurohormones and they were therefore included under this designation. Thus in the activation of color cells the distinction between nervous stimulation and that by neurohormones really disappeared. From this standpoint all excitation of chromatophores, be it nervous or humoral, was believed to be of a single type and dependent upon substances, neurohormones, which from near or far

eventually impinged upon color cells and thus excited them into action. This modern interpretation of chromatophore excitation brought the system of color effectors into line with that of nervous units in general where receptor cells, neurons, and the various types of effectors are believed to be activated in sequence by material excitants which emanate from one unit and act upon the next, thus handing on the nerve impulse from cell to cell. This view of the progressive humoral activation of one unit by another, a view vigorously espoused by such workers as Loewi, Cannon, and especially Dale and associates, though by no means fully established, has proved to be a remarkably successful working hypothesis. From this general standpoint, and yet with chromatic effectors always in view, what can be said of the excitation of nervous units and their appended effectors by what have often been called chemical activators?

In speculating on the transmission of nervous impulses over the interruptions between units in the nervous system, be they receptor cell and neuron, or one neuron and the next, or neuron and effector cell, we are prone to think of the process as time-consuming. To exude a neurohumor from a discharging terminal, to have this neurohumor diffuse across the internuncial space, and to impinge finally upon a receiving neuron or upon an effector and thus stimulate such an element cannot be accomplished instantly. Yet it is well known that in not a few cases these steps must be taken with incredible swiftness. Such instances are clearly illustrated by those repetitious movements in animals that are accomplished in extremely brief intervals of time. In man the repeated tapping of a single finger can be brought about at a rate of somewhat more than 10 beats/second. From the standpoint of neurohumors this implies that the motor nerve terminals must discharge, check, and redischarge exciting neurohumors about once every tenth of a second. Other examples, however, call for even higher rates of activity as, for instance, the scratch reflex of the hind leg of the mouse, 20/second (Williams, Barness, and Sawyer), the beat of the fins of the seahorse, 35/second (Breder and Edgerton), of the wings of the hummingbird, 50 to 75/second (Blake), or again the vibrations of the tail of the rattlesnake, which, according to Chadwick and Rahn, may reach as high as 100/second (Williams, Barness, and Sawyer). Although these rates, all of which are for vertebrates, are relatively rapid, especially those of the rattlesnake tail, they are exceeded by what occurs in the wings of some insects. The movements of the wings of the fruitfly *Drosophila pseudoobscura* were recorded by Reed, Williams, and Chadwick, who used a stroboscopic technique and found that these parts beat as high as 191 double strokes/second. This rate, however, is exceeded by the wing movements in several other insects as mentioned by Wigglesworth: the bumblebee, 240/second; the common bee, 250; the mosquito, 307; the fly, 330.

All these rates, except that for the common bee, date from work of 30 or more years ago. How reliable the technique of these workers was cannot be stated, but if all these earlier records are discarded, there still remains the fully reliable count of 191/second for the fruitfly (Reed, Williams, and Chadwick). Can this remarkably high rate be accomplished by successive discharges of humoral substance at a frequency as high as that of the wing beat? It is such high rates as these that put a strain on neurohumoralism as applied to rapid responses. Even the electrical interpretation of such activities implies in such transfers the passage of charged particles and there can be no more concern in conceiving the rapid passage of chemical activation than in imagining those of particles carrying electric charges. Hence the difficulty introduced by the brief time in which many of these nervous operations are accomplished is a common one to both the electrical and hormonal interpretation of synaptic and other like types of transmission. It must be clear from what has been repeatedly stated in this article that the neurohumoral interpretation of chromatophore activities meets with none of the difficulties that the stimulation necessary for extremely rapid muscle response does. With color cells the most rapid rate of change is a matter of seconds, a time interval ample for internuncial transmission by neurohumors as implied in this modern view of chromatic activation, but how this transmission, electric or humoral, is to be understood for muscle movements, some 200/second, is a question for future speculation. This question, though not directly concerned with chromatophores, is one of the many raised by the study of these color cells, and illustrates how inviting to investigation is this field.

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## CHAPTER X

### Clinical Endocrinology

BY HARRY FREEMAN

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#### I. The Pituitary Gland

The pituitary gland arises from two sources embryologically, the buccal mucosa, which forms the anterior portion, and the diencephalic outpouching, which gives rise to the posterior lobe. The two portions of the gland are distinct entities, morphologically and functionally. The combined structure is enclosed in a bony cap, the sella turcica, covered with a layer of dura mater pierced by the pituitary stalk. The anterior lobe contains two types of cells, the chromophile cells, either eosinophilic or basophilic, and the chromophobe cells, which show no affinity for dyes. The hormonal secretions of the gland are elaborated by the chromophile cells while the chromophobe cells have, as yet, no known endocrine function. The

posterior lobe contains pituicytes, which seem to be the active hormonal elements, and unmyelinated nerve fibers. Its nerve connections with the diencephalon seem to integrate it closely with that structure.

Experimental and clinical work on the anterior lobe shows that this gland influences and in most cases controls all the other glands of internal secretions. There are at present known to be elaborated five separate secretions, the growth hormone, the gonadotrophic hormones, the thyrotrophic hormone, the corticotrophic hormone, and the lactogenic hormone. These hormones generally act through direct effect upon the target organs which they stimulate although in the case of the growth hormone (49) the action is immediately upon the tissues involved. There is a reciprocal relationship between the activity of the various pituitary fractions and of their target organs as shown by hypophysectomy or the administration of large amounts of peripheral hormones. Thus, in the absence of the gonads, the gonadotrophic principles are excreted in excessive amounts while the administration of androgens or estrogens will result in a marked diminution in the values obtained. On the other hand, the functional activity of a given gland depressed by hypophysectomy can be increased by the administration of the specific anterior-pituitary principle. The interrelationships between the anterior lobe and the other endocrines are exceedingly complex since the latter are involved in many phases of metabolism and varying activities of several glands simultaneously are affected. The relationship of the anterior pituitary to carbohydrate metabolism is an example. It has been shown that hypophysectomized animals are exceedingly sensitive to insulin (88) and that experimentally produced diabetes may be ameliorated by hypophysectomy (87). Conversely, diabetes has been produced by the injection of crude anterior-pituitary extracts by Young (185). In addition, the diabetes resulting from pancreatectomy has been ameliorated by adrenalectomy (111) and made worse by the administration of adrenocortical extracts (116). It would seem according to Long (111) and Lukens (116) that pituitary stimulation of the adrenals mediated through the corticotrophic hormone is essential for the maintenance of the diabetic condition in experimental animals. Also, the hypoglycemia of hypophysectomized animals may be due to some extent to the absence of the stimulating effect of the anterior pituitary upon the adrenals. Furthermore, the lack of thyroid stimulation in hypophysectomized animals seems to account for the decreased rate of carbohydrate absorption of hypophysectomized animals since it is restored to normal by the administration of thyroxine (150). The investigations of Price, Cori, and Colowick (136) indicate that the diabetogenic effect of anterior-pituitary extracts is due to an inhibition of hexokinase, which catalyzes the oxidation of glucose and the formation of glycogen.

The growth hormone of the anterior pituitary, unlike the other fractions, acts directly upon the tissues and not through the mediation of the target organ hormones (49). To what extent this action is due to the "metabolic" hormones of the gland (*i.e.*, ketogenic, glycotrophic, nitrogen-retaining, or other factors) is not as yet clear. Its effect upon the skeleton is a stimulation of all the normal processes of osteogenesis at the epiphyseal-diaphysial junction (147). The presence of other hormones is essential for normal growth since young thyroidectomized animals cannot undertake normal growth. Inhibition of growth presumably through depression of the anterior pituitary may result from the administration of large doses of estrogens (187) or androgens (122) although in the latter case small doses may promote growth through stimulation of the pituitary. The use of the growth factor has been of some value in cases of pituitary dwarfism although the lack of availability of pure fractions, their cost, and the production of antihormones limit their present clinical usage.

The thyrotrophic hormone of the anterior pituitary increases the basal metabolic rate of the experimental animal and changes the morphology of the thyroid, resulting in absorption of colloid, hyperplasia, and the appearance of mitotic figures (10). Prolonged administration results in exophthalmos. Its action, however, is relatively brief, presumably owing to the formation of antihormones. In man, hypothyroidism is accompanied by an increase in the amount of thyrotrophic hormone in the blood (82), while in hyperthyroidism the amounts are subnormal (82,160) or the principle is absent. Following thyroidectomy, according to Bodart and Fellinger (16), the usually low thyrotrophic values of the blood of hyperthyroid patients show a rapid increase. On the other hand, De Robertis (37) has noted both low and high values in hyperthyroid cases, the high values being found chiefly in the "ophthalmic type," suggesting a differentiation of hyperthyroidism in two different types. Iodine (4) and thyroid (154) both offset the effects of the thyrotrophic factor.

The anterior lobe also exercises a direct control over milk secretion through its lactogenic hormone, prolactin. Its clinical use has been chiefly restricted to postpartum women with deficient lactation. The evidence is at times conflicting but in the majority of cases its administration increases the amount of milk produced (96,182). In adult animals prolactin has a strong antigonad action (14). In postpartum engorgement of the breasts, the administration of estrogens (99) or androgens (114) decreases the milk production through the inhibition of prolactin.

The dominant influence of the anterior pituitary upon the adrenal cortex was demonstrated by the investigations of Evans (48), who noted adrenal hypertrophy following the administration of anterior-pituitary extract, and Smith (161), who showed atrophy of the adrenals following hypophy-

sectomy with subsequent restoration after injection of anterior-lobe material. Further purification of the extract has shown it to be different from other anterior hormones and through its stimulation of the adrenal cortex to affect end organs and tissues in all ways similar to the administration of adrenal cortex. In mice and rats, White and Dougherty (175) have found that its administration causes an increase in hemoglobin, red cells, and polymorphonuclear leucocytes and absolute lymphopenia. In rats and rabbits, adrenocorticotrophic hormone (ACTH) results in an increase in the  $\gamma$ -globulin fraction of the serum due to the destruction of lymphocytes (176). Dougherty and White conclude that the role of the adrenal glands in combating infection is related to their controlling influence over lymphoid tissue through antibody formation (176). Since a variety of noxious stimuli activate the adrenal cortex it is probable that these first activate secretion of the adrenocorticotrophic principle, which in turn affects the adrenal cortex. The release of adrenaline which accompanies such threatening stimuli is a factor, according to Long (112), in setting off the anterior-pituitary hormone.

The extension of these studies to humans has shown in general a marked agreement in the observations of the effects of ACTH. In a normal young woman injected with 100 mg. daily in divided doses, Mason and co-workers (120) have found an increased excretion of 17-ketosteroids and cortinlike substances, increased amounts of androsterone and etiocholanolone in the urine, and an increase in the carbon dioxide content of the plasma. The blood and urinary cholesterol were decreased. No change was noted in the electrolyte metabolism nor was any effect seen in the erythrocyte and leukocyte counts.

Forsham, Thorn, Prunty, and Hills (53), who have had the most extensive experience with this material, reported on the effects of single injections of 25 mg. in more than 100 patients and normal subjects. The maximum effect was observed within 4 hours. There was a sharp fall in eosinophils in 50 patients with presumably normal adrenals. In addition, there was an increased excretion of sodium, chloride, potassium, and uric acid but no change or decrease in the creatinine excretion. The subjects with normal adrenals could be differentiated from those with Addison's disease by the fact that in the former the eosinophil decrease was very marked (mean 74%) and the uric acid - creatinine ratio was increased (mean 87%). In the latter group the fall in eosinophils was only 4% and the increase in uric acid - creatinine ratio was only 16%.

The administration of 40 mg. ACTH to these normal subjects, daily for 4-6 days resulted in an enhancement of all functions of the adrenal cortex, namely, an increase in 17-ketosteroids, an increase in 11-oxy steroids, retention of sodium and chloride with increased excretion of potassium, and a slight rise in serum carbon-dioxide-combining capacity. There was some

increase in the fasting blood sugar level but no change in the  $\gamma$ -globulin levels. Forsham *et al.* also studied six cases of anterior-pituitary insufficiency and found little response to adrenocorticotrophic stimulation in the severe cases, whereas the subjects with a lesser degree of insufficiency showed evidence of adrenocortical stimulation following injection of ACTH. Clinical usage of the material is restricted, however, by its scarcity.

The gonadotrophic activity of the anterior pituitary has been studied in great detail since its initial demonstration by Smith in 1926. There are two groups of gonadotrophins, (1) those derived from the pituitary directly or derived from the blood and urine of normal humans or women who have undergone menopause, and (2) those obtained from chorionic tissue in pregnant women or mares.

The anterior-pituitary gonadotrophins are of two types, follicle-stimulating and luteinizing hormones. The follicle-stimulating hormone in the female causes growth and development of numerous follicles together with an increase in ovarian weight; thus estrogens are secreted. In the male it stimulates sperm formation (67). The luteinizing hormone in the female causes luteinization and sometimes ovulation on the already ripened follicle. In the male it stimulates the interstitial cells of the testis to secrete androgens. The combination of these two substances results in a synergism the total effect of which is greater than the sum of the increases produced by the two substances separately (45).

The blood and urine of ovariectomized or menopausal women contains considerable quantities of a gonadotrophin which is primarily of a follicle-stimulating type. In women with normal ovarian function a small amount of gonadotrophin is present in the urine during the midinterval (106) which seems similar in nature to that excreted during the menopause (32). Normal males also excrete gonadotrophins of a similar type.

In human pregnancy shortly after the implantation of the fertilized ovum, another gonadotrophin appears. Its detection forms the basis of the Ascheim-Zondek and Friedman pregnancy tests. It increases rapidly during the first two months of pregnancy then declines to relatively low levels, and disappears a few days before parturition. It is demonstrated in large amounts in the placenta and has primarily a luteinizing action. Its action on the human ovary is primarily degenerative in nature (69). In the male it stimulates the interstitial tissue to produce androgen and causes descent of the testis in about 20% of the cases (167). In the pregnant mare a gonadotrophin appears in the blood during the early stages of gestation (44). Its effects seem to be a combination of the responses evoked by human menopausal and pregnancy urine. It has both follicle-stimulating and luteinizing effects, though the quantity of the former is the greater.

The utility of pituitary hormones is limited by a factor of a developing

resistance to their effects over a course of time. This resistance is considered to be in the nature of antibody formation to the hormone protein and is designated as an "antihormone" effect (168). Such resistance can be overcome, at least partially, by discontinuing the medication for several months during which time the "antihormone" effect wears off. It is probable that with purification of the extracts this resistance will be diminished. At present, however, none of the preparations with the exception of chorionic gonadotrophin can be recommended for prolonged usage.

Clinical syndromes of anterior-pituitary dysfunction are relatively few and are related chiefly to disturbances of growth or sexual development. The role of the anterior pituitary in diseases of the thyroid, parathyroid, adrenals, and pancreas is still obscure, although animal experimentation has suggested several possible interrelationships. The conditions described in the following section leave no doubt as to their origin in the anterior pituitary.

#### A. PANHYPOPITUITARISM

Panhypopituitarism, or Simmond's disease, results from total destruction of all elements of the anterior pituitary by neoplastic growth, infectious processes, trauma, or postpartum necrosis (25,145). It occurs at any age, but more commonly in the third to the fifth decades. It is more frequently seen in females. Its characteristic features are asthenia, anorexia, weight loss, amenorrhea, loss of libido and potency, psychic changes (apathy, dullness). Physical findings disclose a marked cachexia, indications of premature senility, loss of axillary and pubic hair, genital atrophy, breast atrophy, low blood pressure, bradycardia, subnormal temperature, and anemia. The characteristic laboratory findings are a depression of the basal metabolic rate, a low fasting blood sugar, sensitivity to insulin (57), "flat" glucose tolerance curves, eosinophilia (47), subnormal 17-ketosteroid (107) and corticoid excretion (173), diminished to absent gonadotrophins in the urine (107,108), and a lack of response to the injection of ACTH (53). In 43% of the cases there are abnormalities in the sella turcica demonstrable by x-ray (47). The symptoms and signs are all explicable on the basis of a failure of secretion of the thyrotrophic, adrenocorticotropic, and gonadotrophic principles.

The condition is often confused with that of anorexia nervosa, a psychological state in which profound malnutrition brings about a similar state of physical symptomatology. A distinction can be made, however, on the basis of the greater youthfulness of these cases, the high incidence of psychiatric disturbances, the lack of x-ray abnormalities in the sella turcica, the preservation of body hair, and the essentially normal levels of 17-ketosteroids, corticoids (173), and gonadotrophins in the urine.

The prognosis in panhypopituitarism is exceedingly grave. As no adequate anterior-pituitary preparations are commercially available, the treatment is limited to the rectification of the peripheral manifestations. Thyroid extract, desoxycorticosterone acetate, salt, and testosterone have been used to increase the metabolism, energy, and muscular strength. The use of thyroid must be attended with caution, however, since rapid elevation of the metabolism may precipitate the individual into an Addisonianlike crisis (108). Frequent feedings are of value in combating the hypoglycemia. More specific therapy, however, must await the availability of better pituitary extracts.

## B. DYSFUNCTIONS OF GROWTH HORMONE

### *1. Underactivity of Growth Hormone*

Underactivity of the growth-promoting factor of the pituitary gland may result in a certain type of dwarfism. The causation of the failure of secretion may lie within the pituitary or be due to pressure from without as by an expanding craniopharyngioma. If other causes for failure of growth can be eliminated, it may be ascribed to hypopituitarism. The net result is a symmetric failure of skeletal development. Such individuals appear extremely immature initially, but later take on the characteristics of premature aging. The mentality is not usually affected. The sexual development appears normal but there is usually amenorrhea in the female and azoospermia in the male. Laboratory data show, as a rule, an increase in the sugar tolerance, increased sensitivity to insulin (57), low 17-ketosteroid output (165), and delayed closure of the epiphyses. The basal metabolism is usually normal and there are no disturbances in water metabolism. X-ray photographs of the sella turcica are normal except when the causative process is an expanding tumor.

Treatment of the condition is not satisfactory. Present preparations of growth hormone are impure and are not recommended for prolonged treatment on account of their high protein content. Recourse must be had chiefly to testosterone (179) and thyroid for their growth-promoting effects. Both of these substances, however, tend to expedite the maturing of the skeleton, which includes the union of epiphyses, and may, in this respect, limit the success of the therapeutic endeavor.

### *2. Overactivity of Growth Hormone*

Excessive secretion of growth hormone accelerates the growth of tissues, particularly that of cartilage, and induces protein anabolism to a supranormal degree. If the disease process begins in the first two decades of life, before epiphyseal closure has developed, a marked increase in general development ensues, known as gigantism. The bony tissues particularly are

affected and there results a symmetrical increase in size with a corresponding thickening of the skeleton. The head becomes large with prominent supraorbital ridges and a protrusion of the lower jaw from overgrowth of the mandible. The skin thickens and wrinkles, the features coarsen, the nose is bulbous in appearance, and the nostrils widen. The external overgrowth is accompanied by splanchnomegaly of all the organs of the thorax and abdomen and a hypertrophy of other endocrine organs. The course of the disease is self-limited. Its progression may be slow or rapid with intervals of remissions and exacerbations. Eventually there is usually a quiescence of activity and the process may stop at any point or there may be a progression to the opposite phase of underactivity as a result of which the subject may exhibit signs of weakness and fatigue and may die from a cardiovascular accident resulting from the prolonged strain upon the circulatory apparatus.

If the excessive secretion of growth hormone occurs after the epiphyses have united the skeletal and soft tissues exhibit changes known as acromegaly. There is a symmetrical thickening of the bones, leading to hypertrophy of the facial skull; there also occur prognathism, enlargement of the clavicles, sternum, and ribs, and hypertrophy of the bones of the hands and feet with exostoses of the phalanges. The skin is coarse, thick, and wrinkled with hypertrophy of the subcutaneous connective tissue and generalized enlargement of the viscera. Again, the condition tends to progress from an active to a quiescent phase. The laboratory findings in gigantism and acromegaly vary with the degree of activity of the disease. In the active stage there is a moderately elevated basal metabolic rate, a decreased sugar tolerance with resistance to insulin, somewhat enhanced 17-ketosteroid and corticoid excretion (173), and decreased excretion of gonadotrophins (presumably due to destruction of the basophilic cells) (102). In the chronic or inactive stage, the findings may be essentially normal. The glycemia and glycosuria may diminish, the metabolism may subside to normal or even low levels, and the 17-ketosteroids and corticoids will decrease to normal values (173).

The treatment of the condition will depend primarily on the presence or absence of a tumor of the pituitary. If there is evidence of an eosinophilic adenoma, surgical removal is indicated. If no tumor is present, the patient is treated symptomatically for whatever complication he may possess, such as diabetes mellitus, hyper- or hypothyroidism, or adrenal insufficiency. Attempts at inhibiting the overactivity of the pituitary by administering large doses of estrogen have not been strikingly successful. X-ray irradiation of the overactive pituitary must be employed with the greatest of caution since all functions of the pituitary may be depressed.

## C. DYSFUNCTION OF GONADOTROPHIC HORMONES

1. *Hypogonadotropinism*

a. *Frohlich's Syndrome.* The most common disorder noted in the function of gonadotrophic hormone is that of the Frohlich's syndrome (adiposogenital dystrophy). This condition is characterized by sexual infantilism, obesity, and frequent disturbances in water metabolism. The genitalia are underdeveloped; axillary and pubic hair is scanty or absent; the testes frequently fail to descend; menstruation is delayed, absent, or irregular; and there may be water retention or excessive diuresis. The mentality is usually normal, but there may be some degree of physical sluggishness, possibly due to the adiposity. The basal metabolism may be slightly depressed and the sugar tolerance is increased. The condition is frequently associated with an expanding lesion in the region of the optic chiasma, a craniopharyngioma or suprasellar cyst, which, in addition to affecting the hypothalamus, compresses or interferes with the innervation of one or both pituitary lobes with corresponding effects on growth and sexual development.

The treatment involves the removal of the tumor (if present), the administration of chorionic gonadotrophins to stimulate the sexual development and complete the descent of the testes, and the employment of a low-caloric dietary regime for the reduction of obesity. It should be remembered that the obesity of children is in the main of nonendocrine origin (163) and that in boys the genitalia may be so embedded in pubic fat that a false diagnosis of hypogonadism is made. In such cases, dietary reduction without endocrine therapy is indicated.

Observations on treatment with chorionic gonadotrophin show that it does not stimulate all testicular functions. Spermatogenic tissue shows little response. Its main effect is on the interstitial tissue as a result of which androgenic material is secreted. Caution must be observed in the treatment so that hypertrophy of the genitalia does not result.

b. *Treatment of Ovarian Dysfunction.* The analysis of the various types of ovarian dysfunction despite exhaustive laboratory investigations is still in the empiric stage. The value of gonadotrophic therapy is still inconclusive. Clinical experimentation has been carried out with chorionic gonadotrophin, equine gonadotrophin, and combinations of anterior pituitary and chorionic gonadotrophin (Synapoidin). Chorionic gonadotrophin alone does not stimulate the ovaries of the nonpregnant woman to develop, to ovulate, or to secrete more estrogen (26). Pregnant mare serum also fails to produce ovulation in the human (18). Sequential and cyclic treatment with equine and chorionic gonadotrophin produces follicular maturation and corpus luteum development in patients with anovulatory ovarian

failure without hypoestrogenism. Patients with hypoestrogenism (with deficient sexual maturation) respond poorly (71). Equine gonadotrophin, alone, has had disappointing results in amenorrhea and oligomenorrhea (1). Synaploidin, according to Davis and Hamblen (34), is inferior in its efficacy to combinations of equine and chorionic gonadotrophins. It is obvious that judgment of this type of therapy must be held in abeyance.

## 2. *Hypergonadotrophinism*

Excessive secretion of gonadotrophic hormones occurs in deficient gonadal activity whether spontaneous or as a consequence of operative removal. There is no evidence that the increase in gonadotrophic hormones has any significance to the individual so far as his comfort or health are concerned. In the menopausal patient, symptoms can be relieved by estrogenic material in insufficient quantity to depress the pituitary so that the relationship between the autonomic disturbances and the degree of functional overactivity of the pituitary is unproved (80). Consequently, there seems no reason for treating hypersecretion of the anterior-pituitary gonadotrophic hormones as such.

*The Neurohypophysis.* The neurohypophysis consists of the posterior lobe of the pituitary, the infundibular stem, and the median eminence. The primary structural elements are the pituicytes, which are derived from the ependymal cells and unmyelinated nerve fibers which arise from the supraoptic and tuberal nuclei, sweep down the infundibulum, and spread out in the neural lobe. The hormonal secretion arises from the intrinsic elements in the posterior lobe, presumably, the pituicytes (61), and is thereupon discharged into the blood stream.

Posterior-pituitary extract exerts striking physiologic effects upon the various systems of the body. The separation of Pitressin and Pitocin from pituitary extracts has allowed an apportionment of the various actions. Pitressin elicits the cardiovascular, renal, and intestinal effects and Pitocin, the oxytocic action. Both substances increase the blood sugar, counteracting the effect of insulin when the latter is given subcutaneously but not intravenously (183) and in the presence of intact adrenal function (22). In man, therapeutic doses of pituitary extract (U.S.P.) or of Pitressin, parentally, result in coronary constriction, decreased cardiac output, anti-diuresis through increased reabsorption of water by the cells of the renal tubules, and contraction of the smooth muscle of the intestine. Pitocin causes increased contractility of the uterine musculature during the later portion of the gestation period and particularly during parturition.

Only one clinical syndrome has been found to be related to the activity of the neurohypophysis. This is diabetes insipidus, a condition characterized by persistent polyuria, with urine of low specific gravity, and by poly-

dipsia, which is secondary to the excessive elimination of fluids. It is due to a loss of, or diminution in, the antidiuretic substance as a result of infections, trauma, tumors, or idiopathic causes which damage the neurohypophyseal area (cf. Chapter VIII).

The treatment consists of the administration of posterior-lobe extract by injection several times daily to inhibit the polyuria. To prolong the effect of the injections the pituitary preparation has been administered in oil (66) or in combination with zinc salts (52). Nasal insufflation of the dry powder has been used with success.

The pressor fraction has been also used clinically in the treatment of paralytic ileus, to diminish postoperative distention. The oxytocic fraction has been used to control postpartum bleeding in the third stage of labor and to prevent or control hemorrhage in therapeutic abortions. Its utilization in the earlier stages of labor has led to hour-glass contractions of the uterus, rupture of the uterus, fetal asphyxia, or even death (36).

## II. The Thyroid Gland

As the primary function of the thyroid is to regulate the oxidative balance of the body, its diseased states may be conveniently classified into three types: those of normal function (euthyroid), those of overactivity (hyperthyroid), and those of underactivity (hypothyroid). The external appearance and size of the thyroid gland are no accurate index of its degree of activity, although, roughly, hyperthyroid states are usually accompanied by some degree of enlargement of the gland and hypothyroid states by some degree of atrophy. Since the thyroid gland is so intimately concerned with iodine metabolism it might be expected that the incidence and type of abnormality in its functioning would be closely related to the iodine content of the soil and water of various regions (117). Other factors, however, enter the picture to affect the geographic distribution: factors of altered hormone balance (menstruation, pregnancy, and hyperpituitarism), diet (goitrogenic substances such as cabbage), and infections.

*Colloid Goiter.* The colloid goiter is an enlargement of the thyroid gland, usually symmetric, occasionally nodular, which generally begins in adolescence. The increase in size is due to an accumulation of colloidal material in the alveoli of the gland. It is most prevalent in certain mountainous regions of the world such as the Alps, Pyrenees, and the Himalayas. In this country it is found chiefly in the areas surrounding the Great Lakes region. It is much less common on the sea coast. Marine and co-workers have shown that its greatest incidence lies in regions where the iodine content of the soil is low and it may be supposed that the constantly renewed supply of iodine from the ocean has acted as a prophylactic factor for this type of deficiency.

The thyroid gland shows, on microscopic section, acini containing low cuboidal epithelium and distended with large quantities of normally staining colloid. The uniform enlargement may be varied by adenomata or cysts with areas of calcification. The enlargement may vary widely in different individuals and may be so great as to press upon the surrounding structures, particularly the trachea, and cause difficulties in respiration.

Colloid goiters are far more frequent in the female sex, possibly because of the hormonal alterations produced by the process of menstruation and pregnancy. Functionally, the gland, despite its abnormal appearance, maintains the oxidative processes of the body at a normal level. The basal metabolic rate, blood cholesterol, and resting pulse rate are all within normal limits. The chief symptoms are those produced by pressure of the enlarged gland on the surrounding organs, causing a feeling of fullness in the neck or respiratory difficulties.

Treatment of the ordinary colloid goiter lies in the administration of small amounts of iodine in any form. Involution of the gland as a result of therapy is more successfully accomplished in the younger than in the older groups. In areas in which the incidence of colloid goiter is high, iodine has been added to the drinking water or the salt of the community. If the enlargement is so great as to be cosmetically undesirable or to cause serious respiratory difficulty, operative removal is desirable.

*Hyperthyroidism.* This condition (Graves' disease) is a constitutional disease resulting from an excessive secretion of the thyroid gland, accompanied by enhanced activity of the autonomic nervous system and characterized by an increase in the basal metabolic rate and its secondary manifestations. It is frequently accompanied by exophthalmos. It occurs commonly in young adults, predominantly of the female sex, and especially in individuals of an unstable temperament. Psychological factors are frequently the important precipitating factors in the onset of the condition and its maintenance despite treatment. The etiology is at present still not certain. There exists abundant evidence that the thyrotrophic hormone of the anterior pituitary has been implicated as the primary cause in view of the fact that its administration in animals results in elevation of the basal metabolic rate and exophthalmos (118, 129). Hormone assays have not as yet definitely settled this problem. In general, investigators (16, 82, 160) have noted low thyrotrophins in hyperthyroidism and high thyrotrophins in hypothyroidism. De Robertis (37) has found low blood thyrotrophins in "classical" Graves' disease and high values in the "ophthalmic type" of hyperthyroidism, thus suggesting two different mechanisms (cf. the discussion Chapters IV and V).

The gland is usually diffusely enlarged to a variable degree. The acini show hypertrophy and hyperplasia with cells of a high columnar type and

small amounts of poorly staining colloid. The actual amount of thyroxine, globulin, and iodine of the untreated gland has been found to be smaller than normal, primarily, it must be assumed, because the secretion is discharged into the circulation as fast as it is formed, with little opportunity for storage.

The symptoms are primarily due to augmentation in the oxidative processes of the body. Heat production is increased, hence the subjects are warm, flushed, and perspire easily. An excessive production of energy occurs so that there is constant muscular movement, restlessness, and tremor of the extremities. Increased combustion results in a steady loss of weight unless the intake of food is increased. The heart beats rapidly even at rest and excessively so under exertion. Cardiac irregularities of the nature of auricular fibrillation occasionally appear. If the process is long continued cardiac hypertrophy may result. Gastrointestinal overactivity with bouts of diarrhea may occur. Muscular weakness and fatigue are common due to the excessive metabolism. Emotional disturbances are frequent and may progress to serious psychiatric disabilities. The exophthalmos, although a characteristic feature, is not an inevitable accompaniment of hyperthyroidism. Either may occur without the other and, indeed, Means (124) speaks of a hyperopthalmopathic phase of Graves' disease rather than the type. As Salter (152) has shown, many exophthalmic cases are in a euthyroid or even hypothyroid state. The eye phenomena noted in Graves' disease are related to lid retraction, to extrinsic muscle weakness, and to swelling of the orbital contents. It is probable that the exophthalmos is due primarily to the last since autopsy dissections of the orbit in 17 cases of Graves' disease (148, 149) have shown that orbital swelling was due largely to increase in fat. From this point of view, the results of thyrotrophic-hormone administration are significant. Within a few hours deposits of fat are found in the extrinsic muscle fibers of the orbit with degeneration and edematous swelling occurring later (135).

The diagnosis may be quite obvious in the typical exophthalmic case but may be quite obscure when the sole symptoms are of a cardiovascular or gastrointestinal nature. Chief reliance in the past has been placed upon the level of the basal metabolic rate and upon the value for blood cholesterol with which it lies in inverse relationship (*i.e.*, high metabolic rate is usually accompanied by low cholesterol). The faults of both of these as criteria have led investigators, notably Salter (152), to investigate the utility of blood iodine as an indicator of the metabolic status of the organism. Salter, Bassett, and Sappington (152) have found that the plasma-bound iodine has a high correlation with the basal metabolic rate in hypothyroid cases showing lower values than 4 gamma per cent and with hyperthyroid cases exhibiting higher values than 8 gamma per cent. In

relationship to the clinical status of the patient, the "hormonal" iodine level was a much more reliable index than the determination of the basal metabolism. This was particularly true in many cases of exophthalmos without hyperthyroidism in which the metabolic level ranged from 20 to 40% but the "hormonal" iodine was within the normal range of 4-8 gamma per cent. Other laboratory findings in this disease are: the high level of blood lymphocytes, decreased 17-ketosteroid excretion (46), glucosuria, hypercreatinuria (100), and hypercalcuria (138). The first two are undoubtedly due to adrenocortical depression while the increased excretion of the others indicate the hypermetabolic effects of thyroxine upon carbohydrate, muscle and bone metabolism.

Hyperthyroidism may result from an activation of adenomatous tissue within the gland. Since the surrounding thyroid tissue is normal an interesting point is raised as to whether the stimulus is within the thyroid or outside of the glands. If the latter it must be assumed that the adenomata are more susceptible to the stimulus than the rest of the gland. The clinical syndrome is no different than that in Graves' disease except that exophthalmos occurs with lesser frequency and intensity. Since all adenomata are potentially toxic and cancer-forming, surgical removal is indicated. In the past the routine treatment of hyperthyroidism was that of subtotal thyroidectomy preceded by a period of iodinization to reduce the basal metabolic rate to its lowest level. Two recent developments have altered many of our previously held concepts in the treatment of the disease: (1) the discovery of antithyroid substances like thiouracil, and (2) the utilization of radioactive iodine.

Thiouracil and its related compounds prevent the incorporation of iodine into thyroxine and diiodotyrosine and thus interfere with hormone synthesis. The failure of hormone production results in excessive activity of the thyrotrophic hormone of the anterior pituitary (140). The net result is a thyroid hyperplasia, loss of colloid, increase in vascularity, together with a variable degree of enlargement of the gland. In these respects, the effects of thiouracillike compounds differ from that of iodine which decreases hyperplasia, increases the amount of colloid, and causes a diminution in the size of the gland. Like iodine, however, the net result is a decreasing metabolic level which is maintained for as long as the drug is administered. The hypometabolic effect is more regular and more stable than that of iodine. With the decrease in the rate of oxygen consumption, the protein-bound iodine (115) decreases, the cholesterol increases (123), and the loss of urinary creatine diminishes. The clinical symptoms regress with the exception frequently of the exophthalmic changes, beginning within 1 to 2 weeks after the administration of the drug.

Thiouracil, of all the related compounds, has been given the widest

clinical trials (144). Initially, its use was considered to obviate the necessity for surgery. It was found, however, to have two objections. In the first place, its discontinuance resulted in a high percentage of relapses into the toxic state, although these were fewer if the drug was continued over a period of a year. In the second place, its application was attended by a small percentage of complications and occasionally by a fatality. The toxic reactions to thiouracil are chiefly dermatitis, fever, leukopenia, and agranulocytosis. The sensitivity of the individual patient to the drug is unpredictable and apparently unrelated to the dosage of the drug or the length of time it is taken. With the exception of agranulocytic changes, the other complications are minor and disappear on omission of the drug. The latter condition is always serious and requires immediate treatment with penicillin and transfusions. The over-all mortality from this complication is 0.5% in a large series, about the same as that from surgery but with the possibility of its arising at any time during the treatment and not in the initial phase of the disease as with the operation. Thus constant supervision is necessitated in patients taking the drug with observations of the white count made at frequent intervals (180).

For these reasons, thiouracil has been used chiefly preoperatively rather than as the sole method of therapy. The increase in vascularity and occasional enlargement of the thyroid gland produced by the drug has caused many surgeons to combine it with iodine, which alleviates these technical objections (141). A new compound, propylthiouracil, has been found to be much safer (11). The occurrence of agranulocytosis after treatment with this drug is exceedingly rare. Its use, without surgery, is chiefly confined to cases in which, because of concomitant conditions, surgery is extremely hazardous. The optimal dosage of propylthiouracil according to Astwood (11) is 50 mg. every 8 hours with a subsequent reduction as the metabolism falls. As with thiouracil the treatment should be maintained for at least a year to minimize the possibility of relapse.

Another form of therapy for hyperthyroidism which may prove the simplest of all is the ingestion of radioactive iodine. Introduced in 1938 by Hertz, Roberts, and Evans (83) as an aid in the study of thyroid physiology, it was later used therapeutically by Hertz and Roberts (84) and Chapman and Evans (23). The activated iodine was initially used in small dosages with additional amounts of ordinary iodide. Later larger doses of radioiodine were used alone. The material is administered, dissolved in distilled water, in a single dose of an average of 40-50 millicuries. The concentration of the activated iodine by the thyroid gland results in a bombardment of the glandular structure, causing a fibrosis of the gland similar to that derived from external radiation, with a resulting hypo-

metabolism. Sustained remissions have been produced in about 80% of the patients treated.

Patients observed over a 5-year period showed no deleterious effects. The  $\beta$ -rays have a short range and whatever activated iodine was not absorbed by the thyroid seemed to be excreted by the kidney. It is nevertheless true, however, that any irradiation therapy is still suspect until our knowledge of the long-term effect is better known.

Irradiated-iodine therapy has been used in some cases of adenocarcinoma of the thyroid. The results to date have not been strikingly successful primarily because according to Marinelli *et al.* (119) only "15% of thyroid cancers may be expected to accumulate iodine to some degree" and therefore to show therapeutic effects. Seidlin *et al.* (155) reported a case of metastatic adenocarcinoma of the thyroid in which the administration of radioactive iodine was successful over a 3-year period in arresting the disease. The induction of uptake of radioiodine in metastases that had previously shown none was ingeniously demonstrated by Seidlin *et al.* (156) by pretreatment with thyrotrophic hormone or by thyroidectomy, the latter procedure removing the competition for the iodine by normal thyroid tissue. Subsequent amelioration of the disease was noted in several cases. The extension of such techniques may lead to a less pessimistic point of view than has been maintained heretofore.

In cases with severe exophthalmos, the problem of therapy is complicated by the fact that rapid diminution in the amount of circulating thyroid hormone, either by surgery or by thiouracil, is not followed by lessening of the exophthalmos but frequently by an exaggeration of the state. It is possible that this phenomenon may be due to the removal of the inhibitory effect of the thyroid on the thyrotrophic hormone (119). It is considered desirable, therefore, to proceed cautiously in the lowering of the metabolic rate, either by making the drug treatment more gradual, by using external or internal irradiation of the thyroid, or, when surgical removal has been accomplished, by maintaining the patient on small doses of thyroid (presumably for its inhibiting effect on the thyrotrophic hormone). When the condition has given rise to keratitis, corneal ulcerations, or ophthalmitis, local surgical measures may be necessary.

*Hypothyroidism.* This is a condition due to a deficiency of the hormonal secretion of the thyroid and may occur from fetal life onward. The fundamental feature of the disease, lowered oxidative balance of the organism, is common in all cases. In adults the condition is called myxedema. In infants, in which special symptoms and signs are noted because of the influence of the thyroid on cerebral and skeletal development, it is known as cretinism.

Myxedema is due to atrophy of the thyroid, destruction by inflammatory

processes (thyroiditis) or operative removal. In the nonoperative types it occurs more commonly in women and after the fourth decade of life. Usually the thyroid gland is small and fibrosed, but it is not uncommon to find mild grades of hypothyroidism in individuals with colloid goiter.

The symptoms are opposite to that of hyperthyroidism. Physical and mental activities are slowed; there is a gain in weight; the skin is rough and dry; the facial hair is sparse; the eyelids are puffy; the speech is slow and thickened. There is a curious "flattening" of the facial expression due partly to the slowed mental processes and to the infiltration of the subcutaneous tissues with a nonpitting type of edema. These patients commonly are sensitive to cold and have paresthesias in their extremities (numbness, tingling, etc.) possibly because of the slowing of the circulatory rate of blood flow. Easy fatigability is common. The heart may be flabby, slow, and show abnormalities in the electrocardiogram. The blood pressure tends to be low and the blood circulation time prolonged. There are abnormalities in menstruation and in both sexes libido is diminished.

The laboratory findings are characteristically a lowered basal metabolic rate, an increased blood cholesterol level, diminished hormonal iodine value (below 4 gamma per cent) (156), microcytic anemia, and decreased 17-ketosteroid excretion (46). It should be kept in mind that there are many patients showing low basal metabolic rates and exhibiting some signs or symptoms of hypometabolism who are not myxedematous (13). Such individuals frequently are under- rather than overweight because of a lack of appetite and are nervous rather than placid due to a state of chronic fatigue.

The treatment for the condition is the administration of desiccated thyroid in dosages varying ordinarily from 0.5 to 3 grains daily. It is preferable to start with smaller amounts and increase the dosage every few weeks depending on the symptoms, heart rate, blood cholesterol, and basal metabolic rate. The symptoms of hypothyroidism are usually markedly improved within a month. The medication, however, is only substitutive and has to be taken constantly.

Cretinism is the condition of hypothyroidism occurring in fetal life or early infancy. Its cause is at present uncertain but may in some cases be due to an insufficient amount of thyrotrophic pituitary hormone. The degree of failure of thyroid secretion is variable. The thyroid gland is usually atrophic and fibrosed. The fundamental features are the same as those in adults but added thereto are the effects on growth and intelligence. The cretinous child, untreated, develops mentally and physically at a slower rate than normal. Skeletal growth is slowed so that centers of ossification are late in appearing and the long bones grow slowly (184). Dentition is delayed and the teeth are faulty. Mentally the degree of

deficiency may vary over a wide range, but the intelligence does not attain a normal level. Clinically the infant eats poorly, is apathetic, has characteristically thick fascies and tongue, dry skin, brittle nails, and a pendulous abdomen, the last from the lessened tonicity of the abdominal muscles. Its expression is moronic or imbecilic and its movements awkward and clumsy. If obtainable, the laboratory findings are similar to those found in adult cases of hypothyroidism.

The treatment again is the constant administration of desiccated thyroid, the dosage varying with the individual child. Recognition of the condition must be early; otherwise treatment will not restore the individual to a normal level. The hormone must be administered throughout life, otherwise the individual will relapse.

The etiology of hypothyroidism is obscure. To what extent the disease is primary in the gland or secondary to a failure of the thyrotrophic principle of the pituitary is unknown. There is, however, a type of hypothyroidism secondary to pituitary failure in Simmond's disease (125), in which the administration of thyroid must be undertaken with extreme caution as fatal results may ensue. It is probable that in such instances the organism is functioning at such a low level due to the atrophy of the thyroid and the adrenals that it is incapable of meeting the stimulation of enhanced metabolic activity.

### III. The Parathyroid Glands

The parathyroid glands regulate the metabolism of calcium and phosphorus. The calcium in the body is confined for the most part (99%) to the bones and teeth. It is excreted through the urine and the feces and, under conditions of pregnancy and lactation, through the placenta and the breast. Since the amount in the body fluids is small and relatively constant, the difference between the calcium intake and output is represented by the calcium deposited or withdrawn from the bony structures. Under conditions of health there is a constant transference of calcium between the blood and skeleton depending on the state of the calcium balance. The normal level of blood calcium is 9–11 mg./100 ml. with a renal threshold of about 7 mg./100 ml. Phosphorus is likewise found largely in the bones and teeth. In the blood its level is 3–5 mg., measured as inorganic phosphate. It also exists in the tissues in organic forms as phosphoproteins, phospholipides, etc., so that the relationship between intake and output is not quite as direct as with calcium.

The parathyroid hormone, according to Albright (7), acts chiefly on the phosphate ions. It causes a marked excretion of phosphates through the kidney; this results in a lowering of phosphate level in the blood serum. As a homeostatic mechanism, phosphorus and with it calcium are resorbed

from the bones in order to maintain adequate blood levels. When the blood calcium has risen to a sufficient level, the excess is excreted through the kidneys. Albright believes that the stimulus for the production of hormone by the parathyroid glands is a serum calcium level below normal. There is little satisfactory evidence, as yet, of the regulation of parathyroid hormone by the anterior pituitary.

*Hyperparathyroidism*, the production of excessive amounts of parathyroid hormone, is usually due to the presence of a single adenoma, rarely multiple adenomas, in one of the four glands or to hypertrophy of the parathyroid tissue. The cause for the latter condition is obscure. The characteristic features of the disease are an elevation of the blood calcium level with a lowering of the blood phosphorus level, together with an increased excretion of both in the urine (6). Whether the stimulation of the hormone acts directly on the bones to increase resorption or on the kidneys to increase excretion is not clear. In any case, if the calcium lost from the body is sufficiently greater than the intake, symptoms and signs appear. These fall into three classes: (a) those due to bone disease; (b) those due to kidney disease; and (c) those due to the hypercalcemia *per se*.

The loss of calcium in the bones is due to the constant resorption so that the bones become softened, show tumors and cysts, and exhibit spontaneous fractures or collapsed vertebrae with signs of pains and aches over the affected areas. When the bony changes are widespread, the condition is called "osteitis fibrosa cystica generalisata."

In the kidney the hyperexcretion of calcium and phosphorus predisposes to the formation of calculi in the pelvis or in the pyramids. The irritation of the kidneys plus the increased amount of calcium in the blood stream leads to polyuria and subsequently polydipsia so that sometimes diabetes insipidus is suspected. The passage of a stone with the characteristic agonizing pain may be the first symptom leading to a suspicion of the underlying disease. The elevated blood and tissue calcium results in its own set of symptoms. Fatigue, drowsiness, decreased neuromuscular excitability, loss of appetite and weight, and constipation are common. Rarely is the level sufficiently high to result in coma and death (35).

A differential diagnosis from other bone diseases is sometimes difficult. Paget's disease is a localized disease with bony structure normal except in the affected areas. The serum calcium and phosphorus levels are normal; the serum phosphatase may be high. Osteoporosis is due to atrophy of disuse or advanced age. The serum calcium, phosphorus, and phosphatase are normal. Osteomalacia is associated with steatorrhea and also shows normal calcium and phosphorus levels; the phosphatase is high. Multiple myeloma usually results in more sharply demarcated lesions in the bones than hyperparathyroidism. The serum calcium can be high

but is usually accompanied by a normal or high serum phosphorus. The serum phosphatase is rarely elevated and Bence-Jones protein is found in the urine in a high percentage of cases.

The treatment of the condition is to remove the adenoma or most of the hypertrophied parathyroid tissue. Most of the bone changes are reversible, exclusive of the cysts. If the kidneys are severely damaged, recovery will not take place. The most serious postoperative risk is the appearance of tetany due to removal of excessive tissue. A diet adequate in vitamin D and calcium salts should be continued for many months.

*Hypoparathyroidism* is usually due to the removal of excessive parathyroid tissue during thyroidectomy. It rarely appears otherwise. The characteristic biochemical findings are the low level of serum calcium and the high level of serum phosphorus. There is no calcium excreted in the urine and the renal excretion of phosphorus is within low to normal limits. The hypocalcemia results in the syndrome known as tetany. The clinical features are the increase in neuromuscular excitability as exhibited by the carpopedal spasm, Chvostek's, Erb's, and Trasseau's signs, laryngeal spasm, and convulsions. In chronic tetany cataracts appear in the lens. In the period of growth the teeth may show aplastic or hypoplastic signs.

Tetany may be due to other causes than hypoparathyroidism. These include inadequate intake of vitamin D (rickets), inadequate absorption of calcium salts and vitamin D in the intestine (steatorrhea), renal insufficiency with phosphorus retention and compensatory lowering of the serum calcium, and alkalosis, due to emotional hyperventilation, excessive vomiting, or the ingestion of large amounts of alkali.

The treatment of tetany due to a low serum calcium is to raise the serum calcium by means of high vitamin D ingestion, most simply by the administration of dihydrotachysterol (5), a highly irradiated ergosterol (85). This substance raises the blood calcium to any desired level and therefore carries with its administration the danger of producing hypercalcemia. This complication can be checked by the routine use of the Sulkowitch reagent (7), which indicates the amount of calcium in the urine and thus, in a rough way, the level of calcium in the blood. If there is no precipitate in the urine, the test is negative for calcium and the blood calcium is probably between 5 and 7.5 mg./100 ml. If there is a faint white cloud, the blood calcium level is probably in the satisfactory range. If there is a heavy precipitate the blood calcium is high.

Dihydrotachysterol is administered by mouth until moderate amounts of calcium appear in the urine. The dosage is initially 3 ml. (15 mg.)/day and with the appearance of calcinuria a maintenance dose of 1 ml. (5 mg.) three times a week or less is kept. Along with this there should be a diet high in calcium intake, a condition easily met by the ingestion of calcium salts in the form of lactate or gluconate.

Thyroid extract (12) raises the blood calcium level and may be of some value in the treatment. Parathyroid extract is not used routinely because of its expense, the pain on injection, and the gradual loss of effectiveness. For acute emergencies, such as the laryngeal spasm or the convulsions, 10 ml. of a 20% solution of calcium gluconate can be given intramuscularly or intravenously or 10 ml. of a 5% solution of calcium chloride intravenously.

For tetanies caused by alkalosis, vomiting, or steatorrhea, the underlying cause must be corrected.

#### IV. The Testes

The fetal sexual tissue appears about the fifth week of gestation around the medial surface of the mesonephros. At first, undifferentiated in character, it begins to develop specific characteristics within the next 2 weeks. The Wolffian (male) and Müllerian (female) structures both appear in each individual. If a testis develops, the Wolffian body develops into the necessary male structures vas deferens, seminal vesicles, etc., and the female structures atrophy. If an ovary develops, the Müllerian structures give rise to the oviducts, uterus, etc., and the male tissue atrophies. Vestigial remnants of the other sex are left in each case leaving each individual to some extent bisexual in character.

In the male the genital tissue develops in such a way that the testis gradually descends through the inguinal canal and during the last month appears in the scrotum. Testicular tissue has two functions: (1) the production of spermatozoa by the seminiferous tubules, and (2) the production of male sex hormone, testosterone, by the cells of the interstitial tissue. Both these functions are under the control of the anterior pituitary; between the latter and the gonadal tissue there is a delicate reciprocal balance (127). The follicle-stimulating hormone initiates the formation of spermatozoa and the luteinizing hormone controls the production of male sex hormone. On the other hand, the maintenance of androgenic activity by the testis results in a partial degree of inhibition of the pituitary. It follows, then, that, in instances of absent or low androgenic hormone secretion, the anterior pituitary is left unchecked and becomes overactive. This point is confirmed by the finding of high values of gonadotrophins in the urine of castrate or eunuchoid males (128).

The activity of the testes can be measured within certain limits by the excretion of androgens in the urine. Both biologic and biochemical methods of assay have been used. The biologic assay has been based on the growth of the comb of the capon and the increase in the weight of the seminal vesicle in the rat or mouse (68). The increase in weight of combs of baby chicks has also been used (55). A colorimetric method of assay was developed by Zimmerman (186). Unfortunately, for diagnostic pur-

poses, the hormonal material is compounded from several steroids with varying degrees of androgenic material, plus a complex mixture of degradation products so that the final value obtained may not be a true index of the production of androgens. The principal androgens found are androsterone and dehydroandrosterone. The latter has about one-fourth the androgenic activity of the former. Androsterone is apparently derived from the testicular hormone, testosterone (20) but about one-third may be derived from the adrenal cortex (38). Dehydroandrosterone may amount to as much as 15% of the total ketosteroids in young adult males (15). The excretion of androgens is diminished after the age of 40 (105). In castrated males and eunuchs there is a marked diminution in the amounts excreted (21). The two compounds also occur in the urine of females, presumably produced by the adrenal cortex.

Males also excrete small amounts of estrogenic substances and even smaller quantities are found in the urines of eunuchs and castrated males, indicating the possibility that some of the estrogen is a metabolic derivative of testosterone. In support of this point it has been found that the administration of testosterone increases the excretion of estrogens in both castrated and normal men (55).

While testosterone is used chiefly for its androgenic effects it influences many other aspects of metabolism. It induces pubertal changes in the male, causing development of the accessory genital structures and the growth of facial and body hair (95); it inhibits spermatogenesis, at least in large doses (77); it inhibits the gonadotrophic and lactogenic hormones of the anterior pituitary (75); it neutralizes some of the effects of estrogens upon the tissues (59); it increases the libido of hypogonadal males and females (2); it results in retention of nitrogen and formation of protein and consequently is a stimulus to growth (95); it elevates the metabolic rate to a moderate degree (95); it causes a retention of sodium and potassium (169). The commonly used preparation is testosterone propionate, which is effective when injected intramuscularly or implanted in pellets. Methyl testosterone is utilized when oral administration is desired. It is similar in its clinical effects to testosterone except that it raises the metabolism more strikingly and increases rather than decreases creatinuria as testosterone does (178). The effects of the administration of testosterone are purely substitutive and regressive effects are apparent after discontinuance of the medication.

Hypogonadism may be due to primary deficiency in the gonadal tissue as following disease, bilateral orchietomy, or trauma to the tissues. It may also be secondary to hypophyseal hypofunction. The distinction may be made on the basis of the high urinary gonadotropins occurring during primary gonadal failure as contrasted with the low values in the secondary

state (72). The differentiation, however, has limited value in view of the relatively inadequate gonadotrophic material available now so that chief reliance must be had to the potent substitutive effects of the gonadal hormone. The clinical features of hypogonadism vary with the degree of deficit in the internal secretion and the age of onset. It is customary to describe two types, one in which the deficiency occurs before the onset of puberty, the other in which it appears after sexual maturity.

Prepubertal deficiency results in the eunuchoid state in which the skeleton shows abnormal length of the long bones with delay in epiphyseal union. The voice is high-pitched; the body and facial hair is scanty and fine; the muscular development is poor; the genitalia are underdeveloped; the prostate is small; libido and potentia are diminished or absent. In postpubertal deficiency, the skeleton is of course not affected. There are alterations in the voice, decrease in energy, decrease in hair growth, frequently a feminine deposition of fat. The genitalia may show evidence of regression. Some degree of sexual drive remains. Spermatogenesis is decreased to absent and the amount of androgenic material in the urine is diminished. There frequently occur vasomotor symptoms somewhat comparable to those of menopausal women.

That there may be varieties of hypogonadism is shown by the syndromes described by Klinefelter, Reifenstein, and Albright in 1942 (103) and that noted by Heller, Nelson, and Roth in 1943 (81). Klinefelter *et al.* (103) described a group of nine subjects from 17-38 years of age with bilateral gynecomastia, aspermatogenesis without aleydigism, and increased excretion of follicle-stimulating hormone. There was good muscular development and relatively normal development of accessory genital structures. Testicular biopsies showed hyalinization of the seminiferous tubules and normal interstitial cells. Therapy with testosterone and progesterone was without success. In the group described by Heller *et al.* (81), the patients showed constantly small atrophic testes, biopsy of which disclosed hyalinization of the tubules and clumping of the Leydig cells, azoospermia, and elevation of the urinary gonadotrophins. Less consistent abnormalities were noted in regard to skeletal growth, hair distribution, pitch of voice, muscular development, and breast enlargement. Here the response to testosterone was good.

The treatment of hypogonadism is in general satisfactorily accomplished by the administration of testosterone (50). Injections of 25 mg. testosterone propionate, intramuscularly, three to seven times a week will in a short time cause an increase in the size of the genitalia, in the appetite, strength, weight, libido, and basal metabolic rate of the subject. Libido will be increased and hair growth of the body augmented. After satisfactory effects have been obtained, the injections can be reduced to a

level where the effects are maintained. If oral medication is preferred, methyltestosterone can be utilized by the sublingual route in dosages about three to five times that of the injected testosterone. To avoid the necessity of constant medication, testosterone pellets have been implanted subcutaneously in the subscapular region, in amounts totaling 300-450 mg., renewed every 3 to 4 months (142).

Children with hypogonadism present special problems. It is undesirable to start them on treatment too early because of the danger of premature closure of the epiphyses (86). Furthermore, the occurrence of precocious development of the genitalia as a result of androgenic treatment is of psychological concern. Finally, the possibility of spontaneous improvement in the condition is always present. For these reasons it seems desirable to postpone treatment until the time when puberty normally starts so that the physiologic and psychological maturations will coincide. In children with delayed growth of pituitary origin, testosterone is a potent stimulus (50). Since no pure growth hormone is commercially available, it may be employed to advantage to accelerate the growth of the long bones in conjunction with chorionic gonadotrophin starting at about the twelfth year. The dose of testosterone should be reduced (5-10 mg. twice a week) to prevent premature closure of the epiphyses. In children with undescended testes, not due to anatomic block, there is divergence of opinion as to whether testosterone should be used. In bilateral cryptorchidism, hormonal function may not be affected since the intra-abdominal temperature is injurious to spermatogenesis but not to the Leydig cells (127). Cryptorchid testes, however, have a higher incidence of tumors than descended testes (62) and the possibility of neoplastic degeneration furnishes an additional reason for the desirability of transferring the ectopic testes to the scrotum. The endocrine treatment of choice is the administration of chorionic gonadotrophin. Results show that about 20% of retained testes responds to this treatment (167). Androgens have been used as an alternative choice on the basis that even if the testes do not descend, the scrotum and cord structures are stimulated to growth so that orchiopexy is more easily accomplished. The results with testosterone, however, have not been too successful (74). In general, treatment should be started early, before puberty has developed and as early as 3 years of age.

The decline of sexual function in older men may result in a sufficient decrease in male sex hormone production to bring about a syndrome comparable to that of the menopause in females. Hot flushes, nervousness, irritability, fatigability, depression, and tachycardia may ensue. The statistical incidence of the male menopause apparently depends to some extent on the interest of the observer, for according to Werner (174) it

is as common as in females, while Thompson (166) states that it is relatively infrequent. At any rate, when other causes for the symptoms have been excluded, testosterone will relieve the symptoms to a marked extent.

Hypogonadism in males secondary to hypopituitarism may be improved by the administration of chorionic gonadotrophin or male sex hormone. In older men in whom the testes are incapable of responding to stimulation, testosterone may be used to relieve the deficiency in testicular function.

In Addison's disease the diminution in adrenocortical functions is accompanied by a diminished production of androgens. Fatigability continues even after adequate administration of adrenocortical extract or desoxycorticosterone acetate. A lack of development of secondary sexual characteristics and deficiency of body hair is frequently observed in males. In such cases testosterone may be used as an adjuvant to the specific therapy (166).

Male sex hormone has been used in Cushing syndrome primarily because of the property of promoting the retention of nitrogen. The negative nitrogen balance observed in these cases may be reversed and the demineralization of the bones improved (8).

Testosterone is of no value or is contraindicated in various conditions in males. Since it tends to produce azoospermia (77), it should not be used in cases of sterility where the spermatozoa count is normal. It was recommended some years ago for cases of benign prostatic hypertrophy. No practical benefit seems to result in the majority of cases, however, and there is also the danger that it may stimulate the development of latent neoplastic growths in the prostate (73). In angina pectoris, Lesser (109) reported subjective improvement in about 50% of the cases with the administration of testosterone propionate, but the less successful results of Levine and Sheller (110) cast doubt on the value of this therapy. In older men with cardiovascular complications, the administration of the hormone may cause sufficient augmentation of activity to precipitate a cerebral or cardiac accident. Its tendency to produce edema of the lower extremities by salt retention may again embarrass the failing circulation of the senile male.

Testosterone has been widely used in a variety of disorders in the female (59). In dysmenorrhea it has been of some transient value in relieving the painful uterine contractions. It reduces the excessive flow resulting from hyperestrinism or at the menopause, relieves premenstrual tension, and inhibits lactation. Curiously enough, it increases the libido of females in cases of frigidity (151). It should be used with caution, however, because of its masculinizing effects when the dosage surpasses 300 mg./month. While most of the effects regress on discontinuance of medication, they are psychologically undesirable.

In recent years testosterone has been used in the treatment of advanced mammary cancer by a number of investigators, notably Adair (3), who has treated 450 cases. The results have been somewhat variable although dramatic improvement has occurred in some instances. In Snapper's (162) 29 cases, castration combined with testosterone did not prevent the subsequent development of distant metastases in cases with axillary involvement at the time of operation. Prudente (137), on the other hand, reported that in his 63 patients treated with radical surgical intervention and testosterone, the instances of 3-, 4-, and 5-year survivals were increased by approximately 100% as compared to a series treated with surgical intervention alone. Cutler and Schlemenson (30) reported on twenty cases treated with testosterone. Twelve showed no significant improvement and eleven of these died within 1 year. Only four patients showed striking improvement but the duration of response was limited. An investigation by the Therapeutic Trials Committee of the Council on Pharmacology and Chemistry (27) concluded that steroid hormone therapy is palliative only. Androgens are of value in patients with metastatic lesions to bones. X-ray studies usually show increased density of bones although sometimes increase of osseous metastases is evident even though the patient is improved symptomatically. Beneficial results are seldom observed on the primary disease or on soft tissue metastases. The relief of pain, the gain in weight, the increased calcification of bones are presumably due to the nitrogen-retaining effects of testosterone. Whether there is an actual inhibition of the lesions is still uncertain. One unfortunate feature of the heavy dosage (300 mg./week for 10 weeks at least) is the masculinization (3). No final decision can yet be made on this form of therapy.

### V. The Ovaries

Like the testes the ovaries are involved in a complicated reciprocal relationship with the anterior pituitary. Under the influence of the follicle-stimulating hormone, the ovaries begin to secrete  $\alpha$ -estradiol. The quantity of estradiol increases to a point at which it inhibits further production of the anterior-pituitary hormone and stimulates the production of pituitary luteinizing hormone. In turn, this hormone results in the extrusion of the ovum from the ovary with the formation of a corpus luteum, which in turn secretes a specific hormone, progesterone. The latter substance continues to be secreted for about 2 weeks if fertilization has not taken place during which the luteinizing hormone is in turn gradually inhibited. In the absence of conception, progesterone production decreases, menstruation occurs and the follicle-stimulating hormone is again secreted, starting the cycle once again.

During this cycle the endometrium undergoes characteristic changes. Under the influence of the ripening ovarian follicle and its secretion of

estradiol, the mucosa increases in thickness and the glandular structures become enlarged, show branching and tortuosity. At this stage ovulation occurs, approximately 14 days after the first day of the previous menstruation. Under the combined influence of estradiol and progesterone the uterine mucosa thickens, the glands become greatly distended, the epithelium is drawn into folds, and the stroma cells become swollen. If the ovum is not fertilized, by the 27th or 28th day, the pregestational endometrium gradually disintegrates down to the basal layer accompanied by a discharge of nonclotting blood, the menstrual flow. In the average woman this sequence is completed in 28 days. Variations of a few days are the rule rather than the exception both in the same individual and in different individuals.

The changes in the endometrium can be followed by endometrial biopsies (139) which have served as a valuable diagnostic tool in the diagnosis of menstrual disorders. Similarly, though less accurately, vaginal smears reflect the variations in the uterine mucosa. Initially the smears show the evenly sized cells of the basal layers with large, oval, deeply staining nuclei. In the last week before menstruation, the cells become more irregular in size and shape, become cornified, and the nuclei become small and pycnotic, showing a tendency to take on the quality of skin.

The metabolism of estrogens is of a complex character. Estradiol is converted to estrone (a reversible reaction), which is subsequently changed to estriol in the presence of progesterone (an irreversible reaction) (134). In the normal woman, there is a biphasic peak of estrogen excretion, one at the midperiod and the other before the menses corresponding respectively, to ovulation and the development of the corpus luteum (159). There is a sharp drop in estrogen excretion preceding the onset of flow. There is considerable variation from month to month in the shape of the excretion graphs. It must be assumed, therefore, that there may be wide variations in the monthly functional activity of the ovary. This is confirmed by the investigation of Brewer and Jones (17) on histologic and cystologic studies on corpora lutea obtained at operation in a series of normally menstruating women. It was found that ovulation occurred most commonly about the middle of the menstrual cycle, but from patient to patient there was a good deal of variation, the range being from the eighth to the nineteenth days after the onset of the previous period.

Estrogen excretion is minimal in female children until the time of puberty (39). In menopausal women, likewise, the estrogen excretion is slight.

In the latter case it may be derived from the adrenal cortex. In the common clinical conditions of ovarian dysfunction, estrogen determinations have not been, as yet, entirely satisfactory in indicating therapeutic measures.

Progesterone secretion is measured by the excretion of pregnanediol, a

metabolic derivative (172). It is present in the normal cycle during the corpus luteum phase and disappears on the occurrence of menstruation. Progesterone is also secreted by the adrenal cortex.

During pregnancy, the total estrogen content of pregnancy urine rises gradually from the first missed period to term. The excretion of pregnandiol has a somewhat different trend. In the first 2 months in which the corpus luteum is the source of progesterone, the values remain at the same level as during the luteal phase of the cycle. After this there is a gradual rise to a maximum in the eighth month with a subsequent fall before parturition. In this second stage, the placenta is the more important site of synthesis (91). Androgens are also found in the urines of women in amounts somewhat less than excreted by normal males (94). They are probably derived mostly from the adrenal cortex.

The administration of estrogens and/or progesterone is followed by bleeding after an interval of 6-10 days and occasionally even during the period of treatment. The decreasing level of the hormone which follows the hormonal sequence during the menstrual cycle is commonly accepted as the cause of the naturally occurring phenomenon of menstruation. It is, however, a complication of therapy in the treatment of menstrual disorders which may at times be undesirable.

The onset of menstruation usually occurs between ages 10 and 17, with a tendency to an earlier menarche in warmer countries. It is initiated by the influence of the anterior pituitary upon the gonadal hormones when the target tissues have arrived at a stage of suitable development. Once the ovarian hormones begin to be secreted marked somatic and psychic changes occur. The secondary sex characteristics show marked development; the pelvis begins to assume the characteristic shape; fat becomes deposited in the hips, shoulder, thorax, and pubis; breast tissue undergoes development; pubic and axillary hair appears; there is a marked spurt in growth; psychosexual attitudes begin to develop. All these features continue until the status of the adult woman has been attained.

Ovarian deficiency in its simplest form is due to a diminution in the supply of estrogens. This is most readily exemplified in the case of the female castrated by operation or by radiation. Menstruation ceases and the uterine endometrium becomes atrophic with degenerative changes in the tubes and vagina. Usually, in 4 to 8 weeks, a variety of symptoms are noted of a circulatory or autonomic nature. The characteristic feature is the appearance of "hot flushes," which are described as sensations of warmth followed by sweating, starting in the trunk and spreading upward over the face. These are rapid in onset, transient in duration, and variable in occurrence. In the severe cases they may occur several times daily. Along with this are frequent complaints of occipital headache, vertigo,

dizziness, fatigability, and tachycardia or palpitations. Psychic disturbances are common and may extend to anxiety states and even frank psychoses, including involutional melancholia. A similar trend of symptoms is noted in the naturally occurring menopause though in a much more variable form. Since the deficiency is a gradual one in contrast with that of artificial menopause, the onset of signs or symptoms is apt to be more insidious. The complaints are usually full blown between ages 45 and 50 though earlier and later onsets are frequently noted. Symptomatic disturbances correlate roughly with cessation of the menstrual flow. They usually antedate it and may persist for years after. There are periods of remissions and exacerbations which may correspond to varying degrees of follicular secretion. Preceding the cessation of menses there are usually periods of irregular menstruation during which there is commonly an onset of symptoms.

The fundamental reasons for the demonstration of the characteristic symptoms are not entirely clear. There is definitely both a deficiency in estrogenic secretion and an excess of gonadotrophic secretion. Which of these two factors is the responsible one is as yet obscure. Heller, Chandler, and Myers (78) administered small (0.5 mg.) daily doses of diethylstilbestrol to 23 oophorectomized women, and observed alleviation of symptoms and estrogenic vaginal changes without any depression of the titer of gonadotrophins. They state that pituitary depression is not essential for reversal of castrational changes and that the increased gonadotrophic secretion is due not so much to overactive production but to failure of ovarian utilization. They conclude that the cause of symptoms is the withdrawal of estrogens. The definitive answer is as yet not available, however, since, presumably under the same endocrine conditions, there may be marked variations in the intensity of symptoms in a given patient. Furthermore, many patients suffer no discomfort or so little that they can be alleviated by psychotherapy or mild sedation.

Whatever the cause, in the well established case, estrogenic material either natural or synthetic (*e.g.*, stilbestrol) does alleviate most of the symptoms and particularly the vasomotor phenomena. The dosage and method of administration depend on the severity of the case. Initially injections may be given to be followed later by oral medication. In the milder cases, oral medication may be sufficient. In every case, an evaluation of the personality structure should be made with a viewpoint to lessening the stresses of everyday life. The duration of the treatment again is a variable depending on the constancy and severity of the symptoms. The menopausal syndrome usually lasts several years, although there may be intervals during which symptoms are mild to absent. Treatment, therefore, must rest on an individual basis and continue with the

smallest amount necessary to control the symptoms without causing untoward effects such as bleeding. There has been no proof that estrogenic treatment results in neoplastic development in the human. The occasional patient who is sensitive to estrogens can be treated with testosterone (59) although the possible masculinizing effects make this type of therapy inadvisable.

Since the most striking evidence of gonadal function in the female is the cyclic phenomenon of menstruation, much attention has been focused upon it and, indeed, the classification of disorders of the ovaries to a large extent based upon the varying disturbances in menstruation. It would seem possible with the scientific techniques at our disposal to make accurate diagnosis of the causal factors in these disturbances. Elaborate studies have been made in various types of ovarian dysfunction of the excretion of gonadotrophins, of estrogens, and of progesterone metabolites. Histologic observations of endometrial mucosa and of vaginal epithelium have been made at frequent intervals (79). Observations on ovulation time by body (33) or uterine temperature (101) or bioelectric phenomena (113) have been pursued over long periods of time. The net result from a clinical point of view has not been of maximum potential value. In addition, disturbances in menstrual rhythm or quantity of flow may result from generalized physical conditions such as obesity, malnutrition, anemia, illness, or psychic factors. Other endocrine conditions, notably those involving the thyroid or the adrenals, may exert a marked influence on the cyclic regularity of this function. In short, the underlying factors of the various conditions may be quite complex and beyond our present abilities both in recognition of the underlying causation and its correct treatment.

The usual classification system of menstrual disorders is largely on a symptomatic and not a causal basis. The subtypes on the basis of interval are: (a) amenorrhea—absence of menstruation; (b) oligomenorrhea—prolonged intermenstrual interval; and (c) polymenorrhea—decreased intermenstrual interval. Variations in the quantity of flow are divided into: (a) hypomenorrhea—scanty flow; and (b) hypermenorrhea—profuse flow. Combinations of disturbances in rhythm and flow are frequently encountered. Finally, where menstruation is painful, the term dysmenorrhea is employed.

Primary amenorrhea refers to a state in which menstruation has never occurred. According to Albright *et al.* (9), primary amenorrheas are grouped into four clinical entities: (1) primary ovarian; (2) menopause shortly before the menarche (with no decrease in stature); (3) panhypopituitarism; and (4) a selective underfunction of one or more gonadotrophic hormones with secondary ovarian atrophy. He described a syndrome which Varney *et al.* (171) had already noted of women with short stature,

retarded sexual development, and high urinary gonadotrophin titers, which both he and Varney regarded as due to primary ovarian failure. These patients resembled pituitary dwarfs but could be distinguished by the high urinary excretion of follicle-stimulating hormone in contrast to the absent excretion in the pituitary cases. The cause of the decreased stature was problematic. They responded to estrogen therapy with development of axillary and pubic hair, growth of breasts, and recurrent uterine bleeding.

The treatment in general of primary amenorrhea has not been satisfactory. Estrogens and gonadotrophins have been employed with a varying degree of success but to date no gonadotrophic material is capable consistently of increasing the size of the ovary or initiating the menstrual cycle. Recurrent bleeding episodes can be produced which are of some value psychically. The generalized development, as has been noted, can be extended into a more adult type.

The treatment of the various types of ovarian dysfunctions is in a somewhat confused state. The induction of menstruation in secondary amenorrhoeic states or the decrease in the interval of oligomenorrhea has been attempted by the use of estrogens (131) or gonadotrophins, both equine (follicle-stimulating) and chorionic (luteinizing) (70), with little success. The diminution of excessive or too frequent bleeding has been essayed by the use of estrogens (131), progesterone (158), androgens (59), and gonadotrophins (34), with varying results. In general, reliance must be placed mainly on the use of estrogens when the flow is scanty or infrequent and on estrogen-progesterone or testosterone-progesterone (64) combinations where it is too frequent or too prolonged. Thyroid gland preparations are of definite value in menstrual disorders when the metabolism is low. In addition, other contributory causes, both general and local, should be investigated and treated.

Dysmenorrhea, likewise, has had a multiplicity of treatments, all effective to a varying degree. The pain can be temporarily diminished in a number of cases by the administration of estrogens throughout the intermenstrual interval. This prevents ovulation and makes the subsequent flow painless (76). The administration of progesterone (51) or testosterone (59) a few days previous to the onset of the bleeding has resulted in some relief. Endocrine treatment is, however, transient in nature and from that point of view questionable in value. It is not even definitely known that the pain is due to intrinsic ovarian dysfunction. In general, treatment should be on constitutional and psychogenic grounds.

Estrogens have been found to be of value in several other conditions. In senile vaginitis the atrophy, drying, itching, and susceptibility to infections have been remarkably improved under their influence. They have

been used to diminish the flow of milk in puerperal lactation (99); their action in this regard is presumably on the basis of an inhibition of prolactin. They decrease the excretion of calcium and phosphorus in osteoporosis (143). Their use in prostatic carcinoma with bone metastases has resulted in some cases in marked palliation of the neoplastic spread due to their inhibiting effect on the androgenic tissue of the gonads and adrenals (89). In carcinoma of the breast with metastases, estrogens have been recommended in postmenopausal cases, particularly when the metastases has involved soft tissue (27). In these cases the amelioration is again presumably due to inhibition of the anterior pituitary, since testosterone has the same effect although more specifically on the bony tissue.

There are several varieties of ovarian tumors which have endocrine effects (60): (1) the granulosa cell carcinoma; (2) the thecoma; (3) the arrhenoblastoma; (4) the adrenal rest tumor; and (5) the thyroid tumor of the ovary.

The granulosa cell carcinoma and the thecoma arise from ovarian mesenchyme and are made up of either granulosal or thecal cells respectively or a mixture. While they have certain morphologic differences their effects are similar. They produce an excess of estrogens with signs and symptoms resulting therefrom. They may occur at any age and the endocrine effects therefore vary with the age of the patient. In children these tumors bring about an early pubescence. Breast tissue becomes enlarged, the uterus is increased in size and pubic and axillary hair appear. Menstruation occurs more or less regularly. In adult women during the reproductive years, the effects are less striking since sexual maturity has been already attained. Disorders in menstruation are the chief symptom. In the postmenopausal woman, uterine bleeding is commonly seen. The uterus is enlarged in response to the abnormal estrogen stimulation. Breast changes may or may not be noted since the capacity of the senile breast to respond is limited. Operative treatment removes the symptoms and in children causes a retrogression of sexual development.

The arrhenoblastoma is a masculinizing tumor. It arises from the rete ovarii, which are vestigial remnants of the male type of tissue and retain some potentiality for development into androgenic tissue. It occurs in young women and results in a sequence of symptoms which may be not only defeminizing but actually masculinizing. Amenorrhea, atrophy of the breasts, loss of feminine contour, hirsutism, deepening of the pitch of the voice, enlargement of the clitoris, all result from the excess of androgen produced. Hormonal assays may show an excess of androgen and 17-ketosteroid urinary excretion but this is not always true (41,132). Operation is followed by regression of the symptoms. Menstruation and breast development soon return. The hypertrichosis, voice changes, and hypertrophy of the clitoris regress more slowly.

A rare tumor of the ovary is that arising from adrenal rests in the ovary. It produces sex reversals similar to that of the arrhenoblastoma.

Combinations of masculinizing and feminizing symptoms may be found in the same tumor. Mechler and Bloch report a case in which there was cyclic menstruation and no atrophy of the breasts or change in feminine contour during the time that hirsutism, voice changes, and hypertrophy of the clitoris were developing. The tumor showed structures resembling both the granulosa cell carcinoma and arrhenoblastoma (126).

The thyroid tumor of the ovary is a rare occurrence. It is made up largely of thyroid tissue, contains iodine, and occasionally may become activated and produce a hyperthyroid state.

## VI. The Adrenal Glands

The adrenal gland consists of two portions, the cortex and medulla, which are of different embryological origins. The cortex arises from epithelial cells arising from the Wolffian body and the medulla from the embryonic sympathetic nervous system (112). Each portion of the gland has different functions and so will be considered separately.

### A. ADRENAL CORTEX

The adrenal cortex is essential for existence, extirpation always resulting in early death. It produces a number of hormones of a steroid nature of which the best known are: (a) desoxycorticosterone, which has to do with the control of water, salt, and potassium metabolism; (b) corticosterone, which affects carbohydrate metabolism by increasing gluconeogenesis and glycogen deposition in the liver, and by decreasing carbohydrate utilization in the tissues; and (c) an androgenic hormone which has to do with protein anabolism and the growth of axillary hair. Growth and kidney function are to some extent affected by the adrenal cortex as is also the deposition of fat (153). The salt- and water-regulating hormone (desoxycorticosterone) conserves water and salt and causes an excretion of potassium. Conversely the administration of salt will reduce the need for desoxycorticosterone (104) and the ingestion of potassium will increase its need.

The adrenal cortex secretes its hormones under the stimulation of the adrenocorticotrophic hormone (ACTH) of the anterior pituitary. It shows a marked response to stress or toxic situations, the "alarm reaction" of Selye (157), characterized by an involution of lymphocytic and thymic tissue, a decrease in circulating lymphocytes and in eosinophiles. The disintegration of lymphocytic tissue into  $\gamma$ -globulin tends to promote the formation of antibodies. These changes are attributable to the cortical fraction. This sequence of events has been demonstrated by White and Dougherty (175) following the injection of ACTH and has served in recent

years as an important point in the diagnosis of adrenal hypofunction (Addison's disease).

The usual indices of adrenocortical function are the urinary excretion of 17-ketosteroids and the 11-oxygenated corticosteroids. The ketosteroids are not exclusively products of adrenal secretion since in men a small percentage (10-20%) represent degradation products of the testis hormone. Both fractions are increased in amount in stress or traumatic situations and both are affected to a varying degree by endocrinopathies of the adrenal cortex. There is, however, no necessary parallelism in their rate of secretions in various diseases indicating the probability that the pathologic processes have differential effects.

In normal subjects there is a diurnal variation of the 17-ketosteroids (133), with a maximal output during the hours shortly after awakening and a steady decline during the day, subsequently reaching a minimum during sleep. This decrement in secretion is constantly interrupted by the stresses and strains of ordinary life (42). The blood lymphocytes which reflect the activities of the corticoid hormone exhibit a converse trend in diurnal variation with a minimum in the morning and a maximum in the late afternoon (43).

### 1. *Addison's Disease*

The single clinical entity of adrenal hypofunction is Addison's disease, described clearly by Addison in 1855. It is a condition in which the adrenal gland shows atrophy or destruction, most frequently due to tuberculosis and generally accompanied by tuberculosis in other areas of the body. It is a relatively rare condition, more common in the male and occurring chiefly between the ages of 30 and 50. Its characteristics are marked asthenia and fatigue, hypotension, gastrointestinal disturbances (*e.g.*, anorexia, nausea, vomiting, and diarrhea), pigmentation of the skin and mucous membranes, acute crises of collapse, circulatory failure, and dehydration. The onset is usually insidious but may occur with an acute collapse. Stress, overexertion, infections, or surgical procedures may at any time precipitate a dangerous state. In the crises there occur a complex of dehydration, hemoconcentration, decrease in blood sodium, retention of potassium, increase in blood urea and non-protein nitrogen, and hypoglycemia.

The symptomatology may to some extent be explained by the functions of the various hormones as outlined above. The asthenia is presumably due to the faulty protein and carbohydrate metabolism from the failure of production of corticosterone. The abnormal salt and water metabolism results from the lack of secretion of desoxycorticosterone. The basis of the gastrointestinal symptoms may lie in the retention of fluid and the

resulting edema of the viscera. The hypotension may be dependent on the disturbances in salt and water metabolism since the blood pressure may be raised by the administration of sodium salts or desoxycorticosterone. For the pigmentation, no definite cause is known. The diagnosis of Addison's disease may be at times difficult and rests on a number of laboratory procedures. The blood levels of sodium are characteristically low, and of potassium characteristically high. The 17-ketosteroid and cortin secretion is diminished (173). Assays, however, are somewhat involved and the simplest techniques have involved tests of the function of the electrolyte metabolism and of the corticoid functions.

The ability to produce diuresis and to concentrate chloride and urea are measured by the Robinson, Power, and Kepler technique (146):

On the evening before the test the patient abstains from ingestion of food and water after 6:00 p.m. He voids at 10:30 P.M. but discards the urine. The night urine volume from 10:30 P.M. until 7:30 A.M. is recorded and the sample saved for further possible analysis. He then voids at hourly intervals from 7:30 A.M. to 12:30 P.M., the volume being noted. At 8:30 A.M. he drinks 20 ml. of water/kg. body weight within 45 minutes. If the adrenal cortex is intact a diuresis is initiated. Thus, if the volume of any 1-hour sample is greater than the night volume, adrenal deficiency is eliminated. If the diuresis does not occur, adrenal insufficiency is suspected and further measures are taken. Venous blood is withdrawn at the end of the test and the urea and chloride is measured. The night sample is also analyzed for the same constituents and the results are calculated by a formula as follows:

$$A = \frac{\text{urea in urine}}{\text{urea in plasma}} \times \frac{\text{chloride in plasma}}{\text{chloride in urine}} \times \frac{\text{largest vol. urine (1 hr.)}}{\text{vol. night urine (9 hr.)}}$$

The value  $A$  is 30 or more in patients without Addison's disease and 25 or less when Addison's disease is present. The exceptions are patients with renal disease.

The cortical fraction of the adrenal cortex is likewise tested by the ability of the adrenal cortex to respond to the stimulus of ACTH as measured by the decrease in blood eosinophiles and the increase in uric acid excretion. The procedure as described by Thorn and co-workers (170) is as follows:

No food is given after 8:00 P.M. on the day preceding the test. On the day of the test 200 ml. water is given at 6:00 A.M., 8:00 A.M., and 10:00 A.M. Urine is collected from 6:00 A.M. to 8:00 A.M. and an eosinophile count is made. Immediately thereafter 25 mg. purified ACTH is injected intramuscularly. Urine is collected from 9:00 A.M. to noon at which time another eosinophile count is made. The absolute decrease of circulating eosinophiles is expressed as the percentage of the initial count. The two specimens of urine are analysed for uric acid and creatinine and the change in the uric acid - creatinine ratio is computed. A fall in eosinophiles of 50% or more of the initial level and an increase of the uric

acid - creatinine ratio to a level above 50% of the control indicate a satisfactory adrenocortical response and in all probability eliminate the possibility of adrenocortical insufficiency. "False positive" reactions may occur due to (1) the fact that the patient may be in acute stress so that the adrenal cortex is functioning at its maximal level and so cannot respond further; (2) decreased renal clearance or (3) an abnormally high production of uric acid, as in gout or leukemia.

Other tests have been suggested, such as the elimination of salt from the diet or the ingestion of potassium. These are both dangerous since they are apt to produce a crisis. Therapeutic tests with cortical hormone, desoxycorticosterone, or salt ingestion may be of value but are frequently inconclusive. The demonstration of calcification (by x-ray) of the adrenal glands is of some confirmatory value.

The treatment of the disease has altered the prognosis from that of a hopelessly fatal state to that of a controllable condition in which the patient, while necessarily under careful supervision, can carry on normal activities to a fair degree. It rests on the continuous administration of desoxycorticosterone and of salt (121). The extract is given intramuscularly daily in doses of 2.5 mg. until a maintenance dose is established. Resort may then be had to the implantation of pellets (92) (125 mg./0.5 mg. desoxycorticosterone acetate required in 24 hours). The implantation lasts almost a year. Salt should be administered daily in dosages of 3-6 g. Since salt and water regulation relieve only part of the syndrome, it may be necessary in some patients to administer whole adrenocortical extract and testosterone to build up the general resistance and maintain a positive nitrogen balance. The dosage should always be carefully supervised since an excess of desoxycorticosterone may lead to edema, hypertension, and cardiac failure, due to the excessive retention of sodium and chloride. In addition the patients' activities should be carefully guided and infections (which are prone to precipitate crises) vigorously treated. The diet should be high in carbohydrate and relatively low in potassium.

In the crisis of adrenocortical insufficiency, whole adrenocortical extract should be administered to remedy the total deficiency. Large amounts (100-200 ml.) may be given intravenously with glucose and salt together with desoxycorticosterone intramuscularly. As the emergency subsides the dosage should be proportionately diminished.

## 2. *Cushing Syndrome*

Hyperfunction of the adrenal cortex is seen in two types of disease entities, the so-called Cushing syndrome (29) and the adrenogenital syndrome. There is a definite relationship between these two conditions, the differences lying chiefly in the fact that in the Cushing syndrome the changes in general are "metabolic" in nature while in the adrenogenital type, the androgenic factors predominate.

The Cushing syndrome is characterized by obesity, amenorrhea, hirsutism, abnormal striae, hypertension, polycythemia, glycosuria, osteoporosis, and a characteristic purple facies. The causal factor was originally ascribed to the presence of a basophilic adenoma. Later Crooke (28) noted hyaline changes in the nontumorous portion of the anterior pituitary which he considered as pathognomonic of the disease. Metabolic investigations of three female cases by Albright, Parson, and Bloomberg (8) demonstrated that the condition was due to an overfunction of the adrenal cortex resulting in hypergluconeogenesis. They postulated that the central phenomenon was the excessive conversion of protein into glucose by the supranormal secretion of cortical hormone. The net result was a protein deficit in the tissues resulting in the muscle weakness, the thin skin, and the osteoporosis (due to lack of bone matrix). The glycosuria, of course, was an inevitable sequence. In addition, the 17-ketosteroid excretion was high, indicating hyperfunction of the cortex. Subsequent investigations have confirmed their views and, indeed, the Cushing syndrome has been found in subjects without basophilic adenomata. The adrenocortical pathology has ranged in this entity from tumors, hyperplastic states, to absence of pathology (97). Whether the original stimulus to hyperfunction of the cortex is extra- or intra-adrenal is not clear. Certainly, in the cases with no demonstrable adrenal changes, the site of the inciting agent is unknown and the hyperfunction may not be accompanied by any anatomic changes detectable by present techniques. It has been suggested by Kepler (97) that the Crooke's changes may indicate a retrograde but not necessarily a degenerative phenomenon secondary to adrenocortical hypersecretion analogous to cytologic changes in the pituitary after castration.

In general, the electrolyte pattern is opposite to that in Addison's disease, with a low blood potassium (24) and an elevation of the plasma chlorides (177). Tests of carbohydrate metabolism show a reduction in glucose tolerance and some degree of insulin resistance (56). The 17-ketosteroid excretion may be normal (173) or high (8). The excretion of cortin may also vary from normal (173) to high (164) levels bearing perhaps some relation to the degree of activity of the condition. The increase in cortin excretion in these cases is not necessarily accompanied by a rise in 17-ketosteroids indicating that these products measure different aspects of adrenocortical function.

The treatment for hyperplasia rests on an attempt to establish a positive nitrogen balance to remedy the defects caused by the hypergluconeogenesis. For this Albright (8) recommended testosterone because of its nitrogen-retaining, calcium-conserving properties (95). He reported in his cases a retention of nitrogen and of phosphorus, a decreasing urinary calcium balance, and a delayed rise in serum phosphatase level (index of bone formation). Along with these, there were noted an increase in strength and

weight, a decrease in the redness of the skin, and a loss of easy bruising. Results by others (31,177) have not been so striking. Investigation should always be made, obviously, for a tumor since in such instances surgical removal results in a cure.

### 3. *Adrenogenital Syndrome*

A related type of hyperadrenocorticism arises from the presence of adrenocortical tumors. These lead to the adrenogenital syndrome which result in the fetus in pseudohermaphroditism, in the infant in precocious puberty, and in the adult in masculinization. The tumors may be benign or malignant and occur more commonly in females. The tumor tissue produces an excess of androgenic hormone which in the male accentuates masculine development and in the female represses the feminizing effect of the ovarian hormones.

Pseudohermaphroditism is marked by the presence of the primary sexual glands of one sex and the secondary sex characteristics of the other. In the female there is an enlargement of the clitoris with atrophy of the ovaries and uterus.

In pubertas praecox, there is premature and excessive development of the gonads with a tendency to masculinization in both sexes. Male children show striking growth and muscular development, early ossification, and marked enlargement of the genitalia with concurrent development of secondary sex characteristics. In females, menstruation may begin early or not appear.

It is sometimes difficult to distinguish this clinical picture in the adult from that of Cushing syndrome or in females from that produced by masculinizing tumors of the ovaries. In general the excretion of 17-ketosteroids is higher in tumor than in cortical-hyperplasia cases. A further point of distinction may be found in the fractionation of the ketosteroids. Kepler and Mason (98) found that, in six cases of tumor, the 17-ketosteroid excretion was 50 mg. or more/day while in cortical hyperplasia the excretion was less than 50 mg. Moreover the  $\beta$ -ketosteroids constituted 50% or more of the total ketosteroids in the tumor cases and less than 50% in the hyperplastic subjects. Johnson and Nesbit (90) found in three cases of adrenal tumor a very high total 17-ketosteroid excretion with  $\beta$  fractions increased 5-15% above the normal.

The treatment is surgical removal. The chief danger is that of a post-operative collapse possibly due to the inhibition of the other adrenal cortex. The crisis can be mitigated by the use of whole adrenocortex extract and desoxycorticosterone-salt therapy. Removal of the tumor may result in a complete disappearance of the abnormal characteristics in the adults. In female children, the prognosis for a normal sexual life is doubtful.

#### 4. *Rheumatoid Arthritis*

New fields for the utilization of adrenocortical compounds were revealed by the successful treatment of cases of rheumatoid arthritis and rheumatic fever by Hench and his co-workers (81a,81b) at the Mayo Clinic. Fourteen patients with rheumatoid arthritis showed dramatic improvement clinically with the administration of 100 mg. of compound E (cortisone) within 48 hours. There was a diminution of swelling and an increase in mobility in the affected joints, a loss of pain, a feeling of well being (amounting to euphoria) and a fall in the sedimentation rate. The return to normality was maintained as long as the medication was administered. Withdrawal of the therapeutic agent was followed by relapse within 48 hours. The administration of ACTH in equivalent amounts produced a similar reversal of the diseased condition to normal in two patients. Three cases of rheumatic fever were also promptly restored to normal health by the administration of compound E.

Comparable results have been noted by Thorn *et al.* (168a) with ACTH and Boland and Headley (16a) with cortisone.

Thorn (168a) has also reported beneficial results in disseminated lupus erythematosus (three patients) and in gouty arthritis.

The treatment, however, has certain limitations. Previous joint deformities still persist despite continued administration. The relief of symptoms continues only during the maintenance of administration of the substances. Severe asthenia follows the withdrawal of medication unless accomplished gradually. Finally, long continued administration of adrenal steroids may lead to the development of Cushing's syndrome (Hills, Forsham, and Finch, 84a).

As yet, the reason for the beneficial effects is unknown. Patients with rheumatoid arthritis do not seem to be deficient in their adrenocortical functioning, at least as measured by the eosinopenic response to either ACTH or epinephrine injection (Thorn *et al.*, 168a). It is possible that in arthritic patients the liver may be abnormally active in destroying 11,17-oxysteroids or may elaborate a different substance so that the normal level of steroid material is insufficient to prevent the onset of the inflammatory reaction.

#### B. ADRENAL MEDULLA

The adrenal medulla consists of chromaffin tissue which acts like modified sympathetic ganglion cells. It is under the control of cholinergic pre-ganglionic fibers arising in the thoracolumbar portion of the spinal cord and secretes adrenaline in response to the activities of these fibers. Adrenaline discharge has been noted in a variety of conditions such as fear,

pain, rage, muscular activity, hemorrhage, hypoglycemia, and the administration of convulsant drugs and anesthetics. A relationship between the effects of these noxious stimuli on the medulla and on the cortex has been claimed by Long in that the stimulus for adrenocorticotropic activity is mediated through the action of adrenaline upon the anterior pituitary (112).

The action of adrenaline may be inhibitory or excitatory; it mimics exactly the effect produced by stimulation of the sympathetic fiber supplying the tissue. Some of the important "sympathomimetic" actions of adrenaline are constriction of arterioles with an elevation of blood pressure, acceleration of the heart rate, contraction of the radial muscle of the iris, salivary secretion, relaxation of bronchial musculature, and inhibition of the small intestine. In addition, it has metabolic effects, such as an increase in blood sugar, lactic acid, and basal metabolic rate. The increase in sugar and lactic acid is due to the mobilization of glycogen in the liver and transformation of muscle glycogen to lactic acid, which in turn is carried to the liver to form more glycogen. This reconversion may account, in part, for the increase in the oxygen consumption rate.

The widespread actions of adrenaline illustrate its usefulness in a variety of disorders. It offers relief in allergic disorders where urticaria or bronchiolar constriction are prominent. In angioneurotic edema of the glottis it may be lifesaving. It is of some value in sudden cessation of the heart beat in drowning, carbon monoxide poisoning, or accidents during anesthesia. It counteracts the hypoglycemic effects of excessive insulin action. It is used in Stokes-Adams disease to increase the ventricular rate. In general the effects are transient, the magnitude and duration depending on the dosage, the site of injection, and the rate of injection. The customary dose is 0.5 to 1.0 mg. (0.5-1.0 ml. of the aqueous 1-1000 dilution) by subcutaneous or intramuscular injection. A more prolonged effect has been obtained by incorporating it in an oily menstruum.

Only one condition of the adrenal medulla has been found of clinical significance, that due to the formation of pheochromocytomas. These are tumors consisting of chromaffin tissue, producing adrenaline, and arising in the adrenal medulla, although occasionally they may have origin in one of the many widespread areas where chromaffin tissue is found during early life (retroperitoneal tissue, ganglions with aorta and vena cava, Zuckerkandl's organ, thoracic sympathetic chain). The tumor is usually single, rarely multiple in origin.

The symptoms presented are commonly paroxysmal attacks of hypertension, with profuse perspiration, tachycardia, palpitation headache, and numbness and tingling in the extremities. Of late it has been recognized that the hypertension may be non-fluctuating and permanent (64,130) so that the differential diagnosis from essential hypertension may be diffi-

cult. In previous years, reliance for recognition of the condition was based mainly on the history and signs of the typical syndrome and the demonstration of the adrenal tumor by pyelogram or perirenal insufflation (19). Recently the utilization of adrenolytic drugs, the benzodioxans (54) which block circulating adrenaline, has afforded a simpler test for the the condition. Of these 1164F, 2-(2,4-dimethyl-1-piperidylmethyl)-benzodioxan, and 933F, piperidylmethylbenzodioxan, have been found most suitable. Goldenberg, Snyder, and Aranow (63) have investigated patients with pheochromocytoma and those with hypertensive disease. The drugs are injected intravenously in a dosage of 10 mg. 933F and 30 mg. of 1164F/m.<sup>2</sup> body surface in a 1 or 2% solution of isotonic sodium chloride over a 2-minute interval. The results show that, in patients with pheochromocytoma, there is a prompt and marked fall in the blood pressure both in the systolic and diastolic phases which lasts for 10-15 minutes. In hypertensive cases there is usually a purely pressor response occasionally preceded by an insignificant fall. In normal subjects there is a mild pressor effect also. The side effects of the drugs are relatively slight and transient. They include sinus tachycardia, flushing, palpitations, nervousness, coldness of the extremities, and hyperpnea.

The treatment of the condition is surgical removal. Following the operation the hypertension subsides and the paroxysmal attacks cease. The test then becomes negative (*i.e.*, there is no fall in blood pressure after the injection of the drugs). If the test is still positive, additional tumor tissue is still present, as noted by Ganem and Cahill (58), when after removal of an intra-adrenal tumor, a subsequent injection of 1164F again produced a significant drop in blood pressure. Another tumor was found which probably originated in a lumbar sympathetic ganglion.

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