"INVESTIGATIONS ON THE EFFECTS OF SOME BIO-CHEMICALLY IMPORTANT COMPOUNDS ON THE ACTION OF INSULIN"

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SUPERVISOR'S CERTIFICATE

Certified that this thesis entitled "Investigations on the effects of some Bio-chemically important compounds on the action of Insulin" - has been revised and rewritten by my student Mr. Mahendra Kumar, M.Sc., under my guidance and supervision, as directed by the Rajasthan University Syndicate at its meeting held on 25th February, 1961 (vide Registrar's letter No. RS.14/57/11725 E, dated 14.3.1961).

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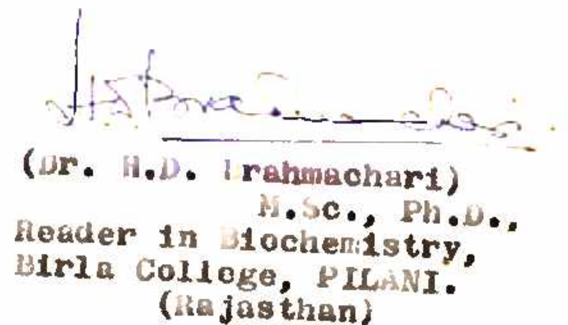

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A B S T R A C T

To elucidate the role of some of the biochemically important compounds on the mode of action of insulin, the present study was carried out. The work has been divided in six parts.

Part I

In this part, effects of tolbutamide feeding on the pancreatic activity of normal guinea-pigs are given. Both the pancreatic amylase activity and the insulin potency increase as a result of tolbutamide feeding for a few weeks.

Part II

The work embodied in this section deals with the effects of sod. beta-hydroxybutyrate on the pancreatic amylase and insulin potency of normal guinea-pigs. It is seen that the insulin potency rises after about 12 days of continuous injections of the drug and again falls after 30 days. On the other hand, pancreatic amylase level goes on decreasing as a result of the injections of this metabolite.

Parts III & IV

The outcome of the preliminary screening tests of different metabolites on the exogenous insulin potency has been incorporated in these sections.

Part V

This part embraces a study of insulin as a surfactant as determined by its effect on the capacity of dropping mercury electrode; further, the effects of different metabolites have been studied on the surface activity of insulin.

Part VI

This portion deals with the paper chromatographic behaviour of insulin alone and as influenced by metabolites.

It is seen in Parts V and VI that insulin behaviour is influenced by the presence of metabolites as depicted by the two properties of insulin made use of in these parts.

INTRODUCTION

The significance of insulin needs no substantiation. The name insulin was first suggested by de Meyer (1909) for the hypothetical internal secretion of the pancreas. A search for the then hypothetical secretion 'insulin' was stimulated by the demonstration of a relationship between pancreas and diabetes mellitus by von Mering and Minkowski (1889). Banting & Best (1922) were the first to achieve a preparation containing insulin. Abel in 1927 obtained insulin in crystalline form and in 1934 Scott showed the presence of zinc in crystalline insulin. The establishment of hypoglycemic property of insulin both in the normal and the diabetic subjects led to the corollary that diabetes was caused by a defeciency of insulin. Presently, this insulin defeciency is believed to be a possible outcome of any of the four ways (Randle 1958): from a defective secretion of insulin by the pancreatic islets, from hormonal antagonism to insulin action, from excessive inactivation of insulin and from some inherent abnormality of cellular metabolism so that the cells are no longer capable of responding to the action of insulin The last possibility is yet of theoretical importance only.

In the annals of endocrinology, no other hormone has attracted so much attention as insulin has and despite the fact that a vast amount of literature on insulin has accumulated, the mechanism of action of insulin is not yet clear.

Duncan (1955) considers diabetes mellitus as a chronic disease manifested by multiple disturbances in the metabolic processes of the body. The greatest apparent disturbance caused

by this disease lies in the defective cellular utilization of carbohydrate which in turn affects the fat and the protein metabolism.

Chaikoff (1951 & 1953) while describing the metabolic blocks in diabetes, confirms the findings of Stetten and Boxer (1944) regarding the failure of the cellular utilization of glucose for lipogenesis. Further, Chernick and Chaikoff (1950) have demonstrated the restoring effect of insulin on the impaired hepatic lipogenesis. The evidence of inter-relationship between glucose and lipid metabolism suggests that the fatty acid synthesis is dependent upon normal glucose metabolism for the production of three substances: (a) Acetyl coenzyme A (acetyl Co-A) which is a structural unit from which fatty acid synthesis proceeds (Lynen, 1952-53), (b) reduced diphosphopyridine TPNH nucleotide (DPNH) which is required in a majority of reductive steps of lipogenesis, (c) reduced triphosphopyridine nucleotide which is necessary for the reduction of crotonyl Co-A to butyryl Co-A (Langdon, 1959). In the light of their findings on the study of the effects of chronic administration of fat metabolites to animals, Nath and Brahmachari (1944, 46, 48, 49) suggested that accumulation of these metabolites leads to the stimulation of pancreatic islets and subsequent exhaustion of the β -cells.

In depancreatized animals, insulin decreases lipemia and cholesterolemia and also prevents the accumulation of large amount of fat which occurs in the livers of untreared animals. The level of ketone bodies in diabetic blood is restored to normal by the

significant results were obtained by insulin injections of very low concentration. On the other hand, Soskin and Levine (1937) and Bouckaert and de Duve (1947) observed that insulin is one of the factors which influence the glycogen-glucose equilibrium in the body; and also it promotes glucose utilization in the peripheral tissues.

Small amounts of glycogen may be deposited in the muscles and livers of depancreatized dogs when no insulin is administered but administration of insulin produces a dramatic increase in the rate of deposition of glycogen both in the muscles and the liver. Stteten's work indicates that insulin deficient animal synthesises its glycogen from three carbon atom chains (lactic acid) but in the normal animal or the one getting exogenous insulin supply, glycogen synthesis takes place from six carbon atom compounds (glucose). Gemmil (1941) showed that the rat diaphragm takes up more glucose for the formation of glycogen in the presence of exogenous insulin.

Observations of Leloir and Cardini (1957) brought to focus a new synthetic pathway for the formation of glycogen wherein the glucosidic linkage of glycogen is formed not by phosphorylase of glucose-1-phosphate but by the transferase from uridine diphosphate glucose (UDGP). In fact later observations by different workers indicate that this may be the major glycogenesis route. Leloir showed that a variety of glucosidic bonds like the one found in glycogen could be generated not only by the elimination of inorganic phosphate from glucose-phosphate but also by the elimination of uridine diphosphate from UDPG, examples of such

formation were shown in the bio-synthesis of trehalose phosphate (1953), sucrose (1953) and sucrose phosphate (1955) by Leloir.

Despite a number of very well documented effects of insulin, the mechanism of its mode of action is not yet clear. A number of theories have been put forward. Recently Stadie(1954) in a review has attempted to analyse all the theories of action of insulin. There can be three important loci where a blocking either by a deficiency of insulin or by an excess of contra-insulin principles would result in a marked disturbance of normal course of metabolism :

1. Most of the glucose metabolism in mammals is initiated by the formation of a hexose-6-phosphate through the action of a specific enzyme which is called glucokinase. The control of the rate of this reaction would necessarily govern the rate of metabolism of glucose. Such type of reactions are called the pace maker reactions (Krebs, 1957).

2. Oxidative reactions of metabolic pathways are connected with the formation and regeneration of adenosine triphosphate (ATP). The total supply of ATP in tissues being limited is quickly exhausted unless constantly regenerated. Since ATP is necessary for the hexokinase reaction and also perhaps for the transfer of glucose across the cell membrane, any qualitative or quantitative defect in ATP regeneration would result in defective carbohydrate metabolism. Also, as all the metabolic pathways are inter-related, any defect in carbohydrate metabolism would influence the course of lipid and protein metabolism.

3. According to another theory, insulin is concerned with the oxidative reactions involving the Krebs' cycle. But this theory is so far ill defined and probably it may be related to the generation of high energy phosphates.

Levine (1949-55) has observed that permeability of certain cells for the entrance of glucose and other sugars is influenced by insulin.

Lundsgaard (1939) and Stadie (1954) have shown that insulin could not stimulate the glucose uptake from cell free systems.

Park _et al_ (1953) have observed that glucose was first transported as such and then phosphorylated.

Whether insulin acts primarily on the transfer of glucose across the cell membrane or its main function is in the utilization of glucose within the cell is still an open question. Although, the theory of insulin action on glucose transport across the cell membrane is most prevalent, yet there is no dearth of well documented insulin effects which could not be explained by this theory. To quote just one such anomaly is Milstein's (1956) finding that insulin directly influences the oxidative rate of glucose-6-phosphate metabolism by phospho-gluconate oxidative pathway.

Condensing enzymes which synthesize citric acid have been suggested as possible sites of insulin action (Beaty and West 1955). Haft _et al_ (1953), Drury and Wick (1951) and Levine (1949-55) have shown that molecular configuration of the sugars conferred some degree of specificity on the action of insulin. Goldstein _et al_

(1953) have observed that the entrance of sugars into the tissues is governed by a transfer system which is specifically adapted with respect to chemical structure. The action of insulin is associated with the utilization of sugars: d-fructose, d-mannose and l-sorbose though metabolised are not as responsive to insulin action as glucose is. These observations support the view that insulin promotes the metabolic transformation of glucose by effecting a rapid entry of glucose into the cell.

Goldstein *et al* (1953) also observed that the barriers which limit the entrance of sugars possessing certain structural forms, into the cell might be overcome not only by insulin but also by humoral agents. Drury and Wick (1951) while supporting the work of Levine *et al* showed that the volume in which glucose could be present intra-cellularly might be a very thin layer underneath the cell surface. Haft *et al* (1953) observed that insulin acts on some system other than the hexokinase system, which facilitates the rate by which sugars enter the cell.

Price *et al* (1945, 46) showed the stimulating effect of insulin on the activity of hexokinase. But Broh-Kahn and Mirsky (1947) reported that the hexokinase activity of muscle extracts from the alloxan diabetic and the normal rats were comparable and addition of insulin had no effect on either of them. Findings similar to those of Broh-Kahn and Mirsky were reported by Stadie *et al* (1949) in cat muscle and by Christensen *et al* (1949) in the erythrocytes of normal and alloxan diabetic rats.

Feller et al (1951) showed that in intact dogs totally devoid of insulin secreting cells, the rate of oxidation of

glucose is far below the normal. But Statten et al (1951) had criticised these findings.

Liver mitochondria have been shown to house a number of metabolic enzymes (Schneider and Potter 1948; Schneider 1948; Kennedy and Lehniger 1949; Potter et al 1951 and Kielley & Schneider 1948).

In alloxan diabetic rats of one year duration, Gerritzen et al (1957) could not trace any complication of diabetes and so these authors were of the opinion that at least in rats, insulin defeciency is not the reason of secondary diabetic complication Bhattacharya (1954) observed that alloxan diabetes was caused by the inhibition of hexokinase activity by alloxan but Villar-Pallasi et al (1957) were not able to support fully such a claim.

Anyway, the question still remains whether insulin acts as such or in combination with different types of metabolites present in the animal systems. It is quite pertinent to ask if the whole picture of diabetes is accounted for by insulin defeciency only .

The present study was undertaken to clarify some of these points not yet clear and was specifically directed to explain the following:

1) To find out any activity relationship between the acinar cells of the pancreas and the beta-cells of islets of Langerhans as influenced by beta-cell-stimulators and by diabetogenic metabolites.

2) Effects of different metabolites on the potency of exogenous insulin.

3) To study insulin as a surfactant by physical methods and the possibility of its forming complexes with metabolites.

4) To study the chromatographic behaviour of insulin and the effect of metabolites on that behaviour.

These findings have been presented in six parts.

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PART I*

STUDIES ON THE EFFECTS OF TOLBUTAMIDE FEEDING ON THE PANCREATIC ACTIVITY OF GUINEA-PIGS

* Results of this part have been briefly published vide Current Science (1959): 28, 214.

A B S T R A C T

Effect of tolbutamide administration for sixtyfour days on the pancreatic activity of normal guinea-pigs has been studied It is noted that both the extractable pancreatic insulin potency and the pancreatic amylase activity increase at the end of this feeding.

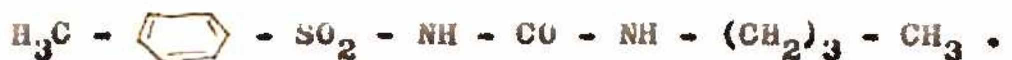
I N T R O D U C T I O N

There had been a constant search for oral substitutes of insulin. The success of the search for an ideal agent which will be effective by mouth in the treatment of diabetes is dependent upon the development of more information about both the mechanism responsible for insulin insufficiency and the mechanism of action of insulin on glucose transport (Mirsky 1958). From time to time, a good number of products have been claimed as oral antidiabetic drugs and recently the products which have proved to be most successful are the sulphonylureas.

Janbon *et al* (1942) during their study on the therapeutic effects of isopropylthiodiazole derivative of sulphanilamide in typhoid fever, noted the hypoglycemic effects of this compound. Subsequently, it was established that this type of compounds were effective in dogs and rabbits only in the presence of 1/10th to 1/5th or more of the pancreas and so it had been suggested that

this type of compounds acted by stimulation of insulin secretion from the beta cells of islets of Langerhans (Loubatiers 1944, 46; Bovet and Dubost 1944). In 1955, Frank and Fuchns, Achelis and Hardebeck and Bertram *et al* described the hypoglycemic action in animals of a new sulphonylurea-carbutamide (1-butyl-3-sulphonylurea, BZ-55, Nadisan 'R', U6987). Shortly after the availability of carbutamide, the Farbwerke Hoechst made available another sulphonylurea which chemically differed from carbutamide in the para position of the phenyl ring and this new sulphonylurea was called Tolbutamide (1-butyl-3-p-tolyl sulphonylurea, D-860, Orinase 'R').

(BZ-55, Nadisan, U6987, Carbutamide)



(D-860, Orinase, U-2043, Tolbutamide)

Elucidation of mechanism of action of sulphonylureas has attracted a number of workers. Colwell and Colwell (1957), Camerini Davalos *et al* (1956) and Wilderberger and Rickettes (1956), all suggest the view that 70 - 80 % of adult type (maturity onset) of diabetics show response to the treatment of sulphonylureas.

Tolbutamide is more effective as a hypoglycemic agent than carbutamide and shows fewer side and toxic reactions (Mirsky *et al* 1956). Mirsky (1958) divides the hypoglycemic response to tolbutamide in normal persons in two phases: (a) an initial phase lasting less than one hour during which a maximum decrease in the blood sugar concentration occurs, and (b) a subsequent phase of restitution lasting a variable period of time during which the blood sugar level is gradually restored to its initial concentration.

It has been shown that the destruction or removal of the beta cells of the pancreas prevents the hypoglycemic action of the sulphonylureas in man (Miller and Craig 1956; Purnell *et al* 1956; Goetz *et al* 1956), dog (Loubatiers 1946; Fritz *et al* 1956; Houssay and Penhose 1956; Mirsky and Gitleson 1957), rat (Mirsky *et al* 1956; Dulin and Johnston, 1957), rabbit (Chen *et al* 1946; Mirsky and Gitleson 1957) and toads (Houssay and Penhose 1956). But in fowls Mirsky *et al* (1957) showed that the presence of pancreas is not essential for the hypoglycemic response to tolbutamide. Thus, in all these species excepting the fowl, action of tolbutamide is dependent upon some factor due to the presence of the pancreas. Mirsky (1958) observed that the initial hypoglycemic response to tolbutamide is possibly due to the stimulation of the beta cells of the islets of langerhans and the discharge of insulin so secreted into the circulation; the prolongation of hypoglycemia is due in part at least, to an inhibition of insulinase and a consequent decrease in the

destruction of insulin. Of course, this hypothesis does not preclude the possibility that additional mechanisms like the inhibition of phosphorylase and of glucose-6-phosphatase may also be involved in the production of these two phases of response to sulphonylureas. Indeed the existence of such additional mechanisms is strongly suggested in the fowl which is independent of the presence of the pancreas for the hypoglycemic response to tolbutamide (Mirsky et al (1957). Tolbutamide feeding does not cause an increase in the peripheral utilization of glucose in man, therefore the stimulation of endogenous insulin by the sulphonylureas has been doubted (Elrick and Purnell 1957; Recant and Fischer 1957; Renold et al 1957). Vaughan (1957) suggests that the hypoglycemia is caused by sulphonylureas by a decrease in the hepatic glycogenolysis.

Volk et al (1957) found that tolbutamide neither inhibited glucagon activity nor stimulated the glucagonase activity. Also, these authors could not observe any effect of tolbutamide on hepatic glycogenolysis or insulinase activity. These workers, therefore, suggested that the action of sulphonylureas could be due to the stimulation of beta cells but at the same time they did not exclude completely any other extra-hepatic action. Dulin and Johnston (1957) also supported the view of beta cells stimulation by sulphonylureas.

Bierman et al (1957) noted that tolbutamide in normal and diabetic persons caused a reduction of blood Nefa of those persons who respond to the hypoglycemic action of tolbutamide. Heinivaara (1956) on the basis of microscopic study suggested the possibility

of some extra-pancreatic mode of action of tolbutamide.

Berson et al (1956) are of the opinion that the lowest concentration of tolbutamide capable of inhibiting the degradation of insulin and glucagon is significantly in excess of the one which is capable of causing the hypoglycemic response in the intact animal.

The above observations lead to the conclusion that the mode of action of tolbutamide is not yet very clear. The work described in this part was carried out to note the effect of tolbutamide feeding on the pancreatic activity of normal guinea-pigs. Effects on the potency of pancreatic insulin and amylase have been reported.

Material and Methods

Four groups of normal healthy guinea-pigs were selected. One group was kept as control and the three groups were fed orally, pure Tolbutamide* powder daily in doses of 100 mg/kg of the body weight of the animal. Throughout the duration of the experiment, animals in all the groups were fed ad lib a diet of green cabbage and germinated grams. Animals had a constant supply of drinking water. From time to time, the animals of different groups were depancreatized and estimations done on pancreas as described under experimental.

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* Presented by Albert David & Company, Calcutta.

Experimental

At regular intervals, animals from each group were depancreatized and pancreas were placed in containers over crushed ice.

A small slice of pancreas was homogenised in chilled distilled water in all glass micro-wet grinder (Town and Mercer). An aliquot of the homogenate so formed was kept in a constant weighing crucible for overnight at 100°- 105° and the weight of pancreas present in the aliquot was found out. The quantitative determination of 'Amylolytic Activity' of pancreas was done by the method of Willstatter, Waldschmidt and Hesse as described by Hawk and others (1954). Briefly the method used was as follows:-

25 ml. of a freshly prepared 1 % starch solution was pipetted out in a Pyrex flask and to this were added 10 ml. of phosphate buffer pH 6.8 (5.1 ml. of 0.2M KH_2PO_4 and 4.9 ml. of 0.2M Na_2HPO_4) and 1 ml. of 0.2 N Na Cl . The solutions were mixed up and brought to 37°C in an electric incubator. 0.1 ml. of the pancreatic homogenate was added and the flasks were returned to the incubator at 37°C. The flasks were kept at 37°C for exactly ten minutes when the enzyme reaction was stopped by the addition of 2 ml. of IN HCl followed by 1 ml. of 0.1 N iodine. To this, sufficient solution of 0.1 N NaOH was added drop by drop, with shaking. This was to neutralize the added HCl and to change the primary phosphate of the buffer to the secondary. For all this 31.5 ml. of NaOH were required. The flasks were allowed to stand for fifteen minutes and then acidified by calculated amount of

dil. H_2SO_4 and the excess iodine was titrated against a standard thiosulphate solution.

The calculations were done on the basis that one ml. of 0.1 N iodine is equivalent to 17.15 mg. of maltose and were expressed by the method of unity (by knowing the dry weight of the pancreas actually presente.. in the homogenate) in terms of mg. of maltose per gm. of dry pancreas.

Extraction and assay of pancreatic insulin
of guinea-pigs was done as follows

Following the method of Jephcott (1931), [illegible] pancreas were minced in cold pestle and mortar with Pyrex glass powder and 4 volumes (for 100 g pancreas) of extraction in liquid, consisting of 75 mls.of ethyl-alcohol, 25 mls. of water and 1.5 mls. of concentrated hydrochloric acid were mixed with it. This mixture was stirred for 2 hours at 37°C after which it was centrifuged at 3,000 r.p.m. till absolutely clear. The residue was again extracted as the original pancreas. Both the clear solutions were mixed and neutralized with conc. NH_4OH with litmus paper as indicator and again centrifuged. In the clear solution sufficient alcohol-ether mixture was added and the solution was cooled till insulin was precipitated. This insulin precipitate was centrifuged and dissolved in distilled water with a trace of hydrochloric acid.

The biological potency of this insulin solution was measured by injecting it to rabbits 1.5 - 2 kg weight and studying the blood sugar fall/hour. Potency of insulin was expressed in terms of blood glucose fall/hour/gm wet weight of pancreas.

Blood sugar determination

Blood sugar estimations were carried out by the following methods:-

1) Preparation of the animals: The ears of the overnight fasted rabbits were properly cleaned of all the hairs, washed with alcohol, dried and rubbed with xylol so that veins were prominent. A little grease was applied on the vein and a prick given on the marginal ear vein with a sharp sterilized needle. Blood was collected directly in the exalated tubes and the sugar estimated by the following methods.

2) Method of Folin and Malmos (1929) was carried out and blood proteins were precipitated by the Haden's (1923) modified method and estimating the blood sugar in the usual way using a Lumetron's Photoelectric Colorimeter (Model EF) at 525 mμ

Statistical Analysis

The data were subjected to statistical evalution and expressed as Mean $\pm$ S.E. S.E. (standard error) was calculated

by the following formulae:

S.D (standard deviation) $= \sqrt{\frac{\sum \text{Square of observations}}{n} - (\text{Mean})^2} \times \sqrt{\frac{n}{n-1}}$

S.E (Standard error) $= \frac{S.D.}{\sqrt{n}}$

n = number of observations.

't' values calculated by the following formulae

S.E.D (standard error of difference) $= \sqrt{\frac{(SD_1)^2}{n_1} + \frac{(SD_1)^2}{n_2}}$

$t = \frac{\text{difference between the means}}{S.E.D.}$

were converted into P values by referring to standard tables (Croxton, 1953) and were considered significant at 5 % level ($P \leq 0.05$)

R e s u l t s

The effects of tolbutamide on the potency of pancreatic insulin are given in Table I.

TABLE I

Effects of tolbutamide on the pancreatic insulin potency of guinea-pigs.

No	Total days of drug feeding.		Insulin potency in terms of blood sugar reduction/hour/g. wet pancreas. (± S.E.)*	Significance of difference
1.	0 Control	(6)	14.72 ± 0.79	-
2.	43	(6)	15.53 ± 3.55	$P > 0.80$
3.	59	(6)	-	-
4.	64	(6)	42.14 ± 3.54	$P < 0.001$

* ± Standard error.

Figures in parentheses indicate the number of animals in each group.

Effects of tolbutamide feeding on the pancreatic amylase activity of normal guinea pigs are given in Table II.

TABLE II

Effect of Tolbutamide feeding on the pancreatic amylase activity of guinea-pigs.

No.	Total days of drug feeding.		Amylase in terms of mg. maltose/g of dry pancreas. ($\pm$ S.E.)*	Significance of difference
1.	0 Control	(6)	654.1 $\pm$ 53.08	-
2.	43	(6)	1688.0 $\pm$ 61.39	$P < 0.001$
3.	59	(6)	1756.0 $\pm$ 52.70	$P < 0.001$
4.	64	(6)	2672.0 $\pm$ 236.67	$P < 0.001$

* $\pm$ Standard error.

Figures in parentheses indicate the number of animals.

D i s c u s s i o n s

Table I shows the response of tolbutamide feeding on the potency of pancreatic insulin of the guinea-pigs. It is noted that as a result of tolbutamide feeding insulin potency of extractable insulin has increased significantly after about 64 days. This could be a result of beta-cells stimulation by this drug. Goldner and Weisenfeld (1958) observe that tolbutamide induced stimulation may ultimately lead to the pancreatic beta-cells exhaustion and aggravation of diabetes. This possible exhaustion of beta-cells by tolbutamide could be more probable in diabetes due to the presence of fewer such insulin secreting cells. So, tolbutamide if to be used for a long time in diabetes should be used with certain amount of caution. ~~only~~.

Table II indicates the effects of tolbutamide on the pancreatic amylase potency of guinea-pigs. The values of amylase also vary in the same fashion as that of insulin potency. This increase in pancreatic amylolytic activity could be explained to be due to an increased proteosynthetic activity of the acinar cells.

So, on the whole, tolbutamide seems to stimulate the pancreas either by the direct action on the cells concerned or indirectly by the suppression of the activity of some anatagonistic factors.

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PART II*

STUDIES ON THE EFFECTS OF PROLONGED INJECTIONS OF BETA-HYDROXY-BUTYRATE ON THE PANCREATIC ACTIVITY OF GUINEA PIGS

*Results of this part have been briefly published vide Nature (1959): 183, 51.

A B S T R A C T

Effect of β -hydroxybutyrate (Sod. salt) injections on the pancreatic activity of normal guinea-pigs has been investigated. Potency of extractable pancreatic insulin increased after twelve days of the regular administration of this drug and again the insulin potency fell after thirty six days. Pancreatic amylase activity steadily decreased as a result of prolonged administration of this drug.

I N T R O D U C T I O N

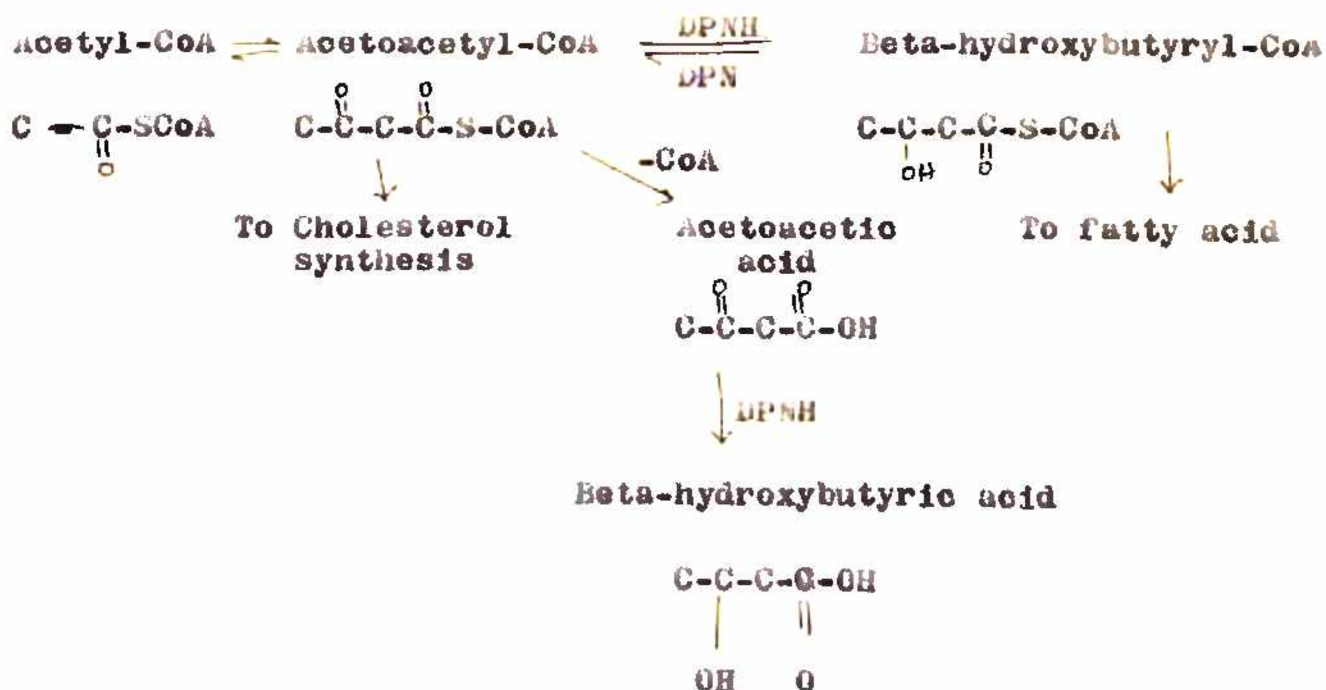
Ketone bodies like acetone, beta-hydroxy-butyrate and aceto-acetate occur to some extent in blood and urine of normal persons. These compounds become more abundant when the carbohydrate stores are deficient and increased amount of fat is utilized to meet the energy requirements of the body. It appears that the large scale ketone bodies production is a secondary consequence to the inability of the body to utilize carbohydrate (Turner, 1955). Ketosis is expected to occur when fats are being catabolized more rapidly than they can be oxidized by the tissues.

One of the important pathways of carbohydrate metabolism influencing lipogenesis is the hexose-monophosphate shunt pathway. It has been noticed by Ashmore et al (1957) that in

diabetes the shunt traffic is lowered.

In diabetes glucose available for metabolism within the cell is greatly reduced and the fraction of glucose utilized via the HMP (Hexose monophosphate shunt) pathway is particularly depressed. Under these circumstances, fatty acid synthesis from acetyl-CoA is limited by the availability of TPNH formed in HMP pathway. It appears that the synthesis of fatty acid is regulated by the glucose utilization via HMP pathway (Shaw and Winegrad, 1959).

The synthesis of ketone bodies begins with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. This compound may lose its CoA to become ketone body-acetoacetic acid or may yield beta-hydroxybutyryl-CoA which in turn becomes the ketone body, beta-hydroxybutyrate (Siperstein 1958). These transformations can be represented as :



It had been shown by Langdon (1955) that both DPNH and TPNH are required for the synthesis of fatty acids in the liver. DPNH is believed to act at the reaction site involving the reduction of acetoacetyl-CoA to beta-hydroxybutyryl-CoA while TPNH is required for the conversion of Crotonyl-CoA to butyryl-CoA. Tchen and Bloch (1957) and also Siperstein (1957) have shown that TPNH is required for the synthesis of cholesterol. Tepperman and Tepperman (1958) observed that the factors that influence the rate of fatty acid synthesis in the liver do not necessarily affect the cholesterol synthetic mechanism in the same direction.

In view of these findings it may be concluded that the major block in diabetes is due to the lack of synthesis of TPNH and the second block is at the conversion of crotonyl-CoA to butyryl-CoA. The location of the lipogenic lesion of diabetes at this site has an important bearing on the metabolic abnormalities as found in diabetes. Diabetic ketosis is characterized by the accumulation of acetoacetic acid and beta-hydroxybutyric acid. Clearly, these acids accumulate, in part at least, as a result of lesion at the TPNH dependent step in the fatty acid synthesis. Because of this metabolic block, coenzyme A derivatives of acetoacetic acid and beta-hydroxy-butyric acid would be prevented from participating in the further synthesis of fatty acids. The concentration of these compounds as a result would increase and their free acids would then accumulate either

by losing coenzyme-A groups or by the conversion of free acetoacetic acid to beta-hydroxy-butyric acid. All these metabolic blocks may be shown as below :

(Blocks have been shown by thick arrows)

Dole (1958) thinks that probably ketonemia also requires an accelerated transfer of fatty acids from depot to liver tissue. This may be due to an increase in NEFA level in the diabetic blood.

From the above observations it is concluded that any increase in the amount of ketone bodies in the system is only a result of certain metabolic blocks. Koehler et al (1941) have shown that the injections of fat metabolites lead to a great

increase in the acetone bodies of the blood of normal subjects. Nath and Brahmachari (loc. cit.) observed that continued injections of beta-hydroxy-butyrate in animals could bring about hypertrophy of beta-cells which may be followed by the destruction of these cells. So, it was thought interesting to study the pancreatic response of normal guinea-pigs to an artificially maintained high level of Sod. beta-hydroxy-butyrate which was injected in aqueous solution.

Experimental

Normal guinea-pigs were divided into four groups. One group was termed as the control group and the other groups were given daily injections at the rate of 50 mg/animal of Sod. beta-hydroxybutyrate (L. Light & Co.) dissolved in sterilized normal saline in the morning before giving food. The dose of this drug was increased every week by 10 mg/animal. Food of the animals in all the groups consisted of green grass, cabbage leaves and germinated grams. Animals in control group were injected only sterilized normal saline. From time to time, animals of different groups were depancreatized and pancreatic insulin and amylase potency estimated as described under Part I.

Results

Results of effects of Sod. beta-hydroxy-butyrate (L. Light & Co.) on the amylase contents of normal guinea-pigs. pancreas are given in Table III.

TABLE III

Effects of Sod. beta-hydroxybutyrate
on pancreatic amylase of guinea-pigs.
.....

No.	No. of days of injections.	Amylase in terms of mg. maltose/g. dry pancreas. (± S.E.)*	Significance of difference.
1.	0 Control (6)	654.1 ± 53.08	-
2.	12 (6)	596.7 ± 7.33	P > 0.30
3.	25 (6)	457.2 ± 34.30	P < 0.05
4.	36 (6)	100.8 ± 13.09	P < 0.001

Figures in parentheses indicate the number of animals.

* ± standard error.

Effects of these injections on the pancreatic insulin potency are given in Table IV.

TABLE IV

Effects of beta-hydroxy-butyrate injections on the pancreatic activity of guinea-pigs.

.....

No.	No. of days of injections.		Insulin potency in terms of blood sugar reduction/hr/g wet pancreas (± S.E.)*	Significance of difference.
1.	0 Control	(6)	14.72 ± 0.79	-
2.	12	(6)	86.10 ± 3.31	P < 0.001
3.	25	(6)	-	-
4.	36	(6)	30.60 ± 2.19	P < 0.05

Figures in the parentheses indicates the number of animals.

* ± standard error.

D i s c u s s i o n

From Table III the effect of Sod. beta-hydroxy-butyrate on the amylase level of pancreas is clear. An inspection of this table reveals the lowering in the amylase activity of pancreas in the normal guinea-pigs. It is evident from this table that as the number of days of this metabolite injections increases, the amylase activity goes on decreasing. This is a significant effect as in diabetes digestive complications are often noted. It appears that beta-hydroxy-butyrate possibly lowers the protoesynthetic activity of the pancreas.

Table IV shows the effects on insulin potency. The pattern of results is similar to the one reported by Nath and Brahamachari (loc. cit). The initial rise in the insulin activity could be due to direct or indirect stimulation of beta-cells of islets of Langerhans by Sod. beta-hydroxybutyrate. As this increase in the insulin potency is followed by a marked fall it is possible that the hypertrophic stimulation of beta cells is followed by a sort of overwork atrophy.

It is possible that beta-hydroxybutyrate in some way inhibits the potency of insulin which leads to an increased insulin demand by the body and thereby, causing pancreas to overwork.

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PART III

EFFECTS OF SOME METABOLITES ON THE POTENCY OF EXOGENOUS INSULIN *IN VIVO* AND *IN VITRO*

ABSTRACT

Preliminary data on the hypoglycemic effects of exogenous insulin as effected by Sod. beta hydroxybutyrate, Sod. Succinate, Sod. Citrate and Sod. iso-citrate has been presented. It is seen that excepting beta hydroxybutyrate, all the metabolites either prolong or enhance the action of insulin.

INTRODUCTION

As a result of metabolic blocks in diabetes, fat metabolites like beta-hydroxybutyrate accumulate in the system. Concentration of such fat metabolites governs the important clinical and biochemical symptoms of ketosis, acidosis and ultimately diabetic coma.

In diabetes, while there is a rise in the concentration of fat metabolites, Frohman and Orten (1955) have demonstrated a decreased level of tricarboxylic acid cycle intermediates.

The chief mechanism by which the four carbon atoms intermediates of Krebs cycle are formed, is believed to be by fixation of carbondioxide to either pyruvate or phospho - enolpyruvate which yield malate or oxalacetate respectively. The formation of malate as shown by Ochoa et al (1948) requires TPNH as a co-factor. It is reasonable, therefore, to conclude

that the depressed four carbon atom compounds of the tri-carboxylic acid cycle in diabetes may be a secondary consequence of the lack of TPNH.

Cruickshank _et al_ (1957), Thompson (1946) and Baron _et al_ (1946) have described the stimulating effects of some of the metabolites on the oxygen uptake of skin. Cruickshank _et al_ (1957) found that succinate could cause an initial stimulation of skin for the oxygen uptake. According to Bullough (1954) succinate and citrate produced mitotic activity in mouse-ear skin _in vitro_ and this activity is maximal in four hours. In the light of the findings of the authors mentioned above and also of Griesmer and Gould (1954), it had been suggested that T.C.A. cycle may not be existing in its classical form in the skin but anyway, it stands that succinate could stimulate the uptake of oxygen while citrate could initiate at least a transitory stimulation.

Beatty _et al_ (1955) observed that metabolites like succinates could reduce the ketone body production by liver _in vitro_.

The recent discovery that a large number of metabolic enzymes are associated with mitochondria (Green, 1951) has initiated a new thought in the mode of action of insulin. According to Green _et al_ (1948) and Green (1952) mitochondria may be considered as the physical housing of a complex of a large number of enzymes which have been called cyclophorase complex enzymes. This group of enzymes viz. cyclophorase-mitochondrial system is called C.M. system. Since the activity

of these enzymes would depend upon the availability of substrates in the cells, any change in the concentration of these metabolites may bring forward some interesting consequences. Nath and Brahmachari (loc. cit.) noted the inactivating influence of some of the ketone bodies on the potency of exogenous insulin. Recently, Beatty and West (1955) reported that the administration of succinic acid with insulin caused a larger decrease in ketonuria than when insulin alone was given. But there is so far no comparative work done on the effects of metabolites on the potency of insulin. And so it was planned out to study the effects of some of the metabolites on the potency of exogenous insulin on pilot scale.

Metabolites studied were :

i) Sodium beta-hydroxybutyrate.
ii) Sodium succinate.
iii) Sodium citrate.
iv) Sodium Isocitrate.

Experimental

a) Preparation of solution for 'in vivo' work

Requisite amount of the metabolites were weighed and dissolved in normal saline. These were injected simultaneously with the standard crystalline zinc insulin (Lilly) in the

different legs of rabbits which had been kept fasting for 18 - 24 hours.

b) Preparation of solutions for in vitro work

For in vitro work 5 mg. of the metabolite were added to a standard solution of insulin just before injecting it in the legs of fasting rabbits.

c) The effect of insulin (control) on the depression of blood sugar level of the rabbits was studied by injecting 2 units of insulin per rabbit of about 1.5 - 2 kg. wt. This solution of insulin 2 units/ml. was prepared just before use by suitably diluting a standard sample of 40 units/ml. insulin (crystalline, zinc) of Lilly with normal saline.

d) The samples of blood from the marginal ear veins of rabbits were obtained in the usual way and blood sugar estimated by the method of Folin and Malmros (1929) as mentioned in earlier part. Blood glucose levels in rabbits were followed for four hours.

Results

As this work was undertaken as a preliminary for a future large scale screening programme, full statistical analysis had not been carried out in this portion (No. of animals being 4 every time)

The values of the depression in the fasting blood sugar level of rabbits as influenced by the different metabolites are given in Table V . (Only average values are given in these tables.

<u>T A B L E V</u>

Effects of 2 units insulin on the glucose level/100 ml. of blood of the normal fasting rabbits.

No.	Time after injection.	Average value of blood sugar level/100 ml. of whole blood. ± SE
1.	0 (Fasting)	100.1 mg. ± [illegible]
2.	1 hour.	52.25 mg. ± 6.1
3.	2 hours.	56.4 mg. ± [illegible]
4.	3 hours.	60.1 mg. ± [illegible]
5.	4 hours.	62.5 mg. ± 6.1

TABLE VI

Effects of beta-hydroxybutyrate (Sod. Salt) on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

No.	Time after injection.	Injection of beta-hydroxybutyrate only (100 mg.)	100 mg. beta-hydroxybutyrate and 2 units insulin (*in vivo*)	.5mg. of drug in 2 units insulin (*in vitro*)
1.	0 (Fasting)	88.54 mg.	83.4 mg.	96.20 mg.
2.	1 hour.	88.54 mg.	82.95 mg.	87.42 mg.
3.	2 hours.	83.51 mg.	-	78.07 mg.
4.	2 ¼ hours	-	95.92 mg.	-
5.	2 ¼ hours.	-	93.07 mg.	-
6.	3 hours	88.54 mg.	92.0 mg.	-
7.	3 ½ hours	-	-	78.07 mg.

T A B L E VII

Effects of Sod. Isocitrate on the potency of Insulin (2 units) in terms of B.S. level/100 ml. of blood of the normal fasting rabbits.

......

No.	Time after injections	Effect of 100 mg. Sod. iso-citrate only.	100 mg. of isocitrate & 2 units insulin (in vivo) (±S.E)	5 mg. of isocitrate & 2 units insulin (in vitro) (±S.E)
1.	0 (Fasting)	79.2 mg. [illegible]	72.61 mg. +7.2	97.5 mg. [illegible]
2.	1 hour.	65.9 mg. ± 5.5	44.53 mg. [illegible]	46.09 mg. [illegible]
3.	2 hours.	65.9 mg. + 6.5	32.14 mg. [illegible]	43.74 mg. [illegible]
4.	2½ hours.	65.0 mg. [illegible]	-	-
5.	3 hours.	-	27.18 mg. ± 2.[illegible]	60.38 mg. [illegible]
6.	4 hours.	-	-	69.1 mg. [illegible]

TABLE VIII

Effects of Sod. citrate on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

.....

No.	Time after injections	Effects of 100 mg. Sod. citrate only	100 mg. of citrate & 2 units insulin (*in vivo*) (± S.E)	5 mg. citrate and 2 units insulin (*in vitro*)
1.	0 (Fasting)	85.02 mg. ±6.4	99.54 mg. ±9.[illegible]	89.40 mg. ±5.1
2.	1 hour.	98.83 mg. [illegible]	26.44 mg. [illegible]	32.68 mg. ±3.3
3.	1 hour 33 min.	-	35.77 mg. [illegible]	-
4.	2 hours.	82.31 mg. ±8.1	-	30.80 mg. ±3.1
5.	2 hours 58 min.	-	44.55 mg. [illegible]	-
6.	3 hours.	87.74 mg. ±8.1	-	-
7.	3¼ hours.	-	-	24.04 mg. [illegible]
8.	4 hours.	88.48 mg. [illegible]	60.30 mg. ±5.5	-

TABLE IX

Effects of Sod. succinate on the potency of insulin
(2 units) in terms of glucose level/100 ml.
of the normal fastings of rabbits.

......

No.	Time after injections.	Effects of Sod. succinate (100 mg.).	100 mgs. succinate & 2 units insulin (*in vivo*) (±5.5)	5 mg. succinate and 2 units insulin (*in vitro*)
1.	0 (Fasting)	64.73 mg. ±5.5	80.50 mg. ±7.1	69.21 mg. ±5
2.	1 hour.	76.10 mg.	38.99 mg.	34.00 mg. ±2.5
3.	1 hr. 25 min.	-	36.03 mg.	-
4.	1 hr. 35 min.	72.70 mg. ±6.5	-	-
5.	2 hours.	-	-	30.96 mg.
6.	3 hours.	61.31 mg. ±5.4	30.10 mg. ±2.5	-
7.	3 hrs. 10 min.	-	-	34.00 mg.
8.	4 hours.	-	-	50.70 mg.

Discussion

An inspection of these tables reveals that the effect of insulin in the presence of metabolites is either enhanced or prolonged, excepting in the case of Sod. beta-hydroxybutyrate (Table VI) which inhibits the effect of insulin on blood sugar levels of fasting rabbits both *in vitro* as well as *in vivo*.

As T.C.A. cycle is on the whole depressed in diabetes, this potentiation of insulin is of great importance. Probably the increase in the concentration of these metabolites which are substrates for the enzyme system of mitochondria (C.M. System), enchances the activity of those enzymes and the glucose uptake of the cells increases. Increased availability of these metabolites of T.C.A. cycle may serve as acceptors of amino-nitrogen and thereby increasing the rate of glucose utilization. It is also possible that these metabolites may form a certain type of complex with insulin which is on the whole more potent than insulin.

Sod. beta-hydroxybutyrate was shown by Nath and Brahmachari (loc. cit.) too, to inactivate the insulin activity and here again this type of inactivation is noticed.

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PART IV

EFFECTS OF INSULINASE INHIBITORS AND SOME AMINO ACIDS ON THE POTENCY OF EXOGENOUS INSULIN

A B S T R A C T

Results of preliminary screening survey of the effects of different insulinase inhibitors and amino acids on the potency of exogenous insulin have been reported.

I N T R O D U C T I O N

Protein utilization in the animals under diabetic conditions is of great significance. It had been noticed quite a long time back that proteins can be replaced in the diet by their hydrolytic products - amino acids. In fact the importance of proteins in the diet is due to their being a source of amino acids only. It is interesting to note that quite a good number of workers have noted that insulin can cause a reduction of the plasma amino acids (Luck and Morse 1933; Frame and Russell 1946; Harris and Harris, 1947 and Milman et al 1951). This reduction in plasma amino acids has been considered by Munro (1951) and Russell (1955) as an evidence of an intimate inter-relationship of increased utilization of carbo-hydrate with a net gain in the metabolism of protein.

Rubini and Seligson (1958) recently confirmed the acute reduction in the plasma amino acids of normal persons by measures which increase the carbohydrate utilization.

Mirsky _et al_ (1956) noted that the feeding of 1-tryptophan could cause a decrease in blood sugar levels of normal rats and mice and they accounted this by suggesting that 1-tryptophan causes an inhibition of insulinase activity. Mirsky _et al_ (1957) further found that this inhibition of insulinase could be caused by even the metabolic derivatives of 1-tryptophan. Among the most important of these metabolites are nicotinic acid and indole-acetic acid.

Mirsky (1956) suggests that the auxins or plant growth regulators like Indolyl-3-butyric acid and Indolyl-3-propionic acid are also competitive reversible insulinase inhibitors. Mirsky (1957) also found that even though 1-tryptophan was quite a potent insulinase inhibitor, D-tryptophan was not, and so also other amino acids like aspartic acid, alanine and phenyl-alanine which were found to be ineffective as insulinase inhibitors.

At the same time the importance of amino acids for the transformation into keto acids, by metabolic deamination and thereby entering into the T.C.A. cycle cannot be exaggerated.

Aspartic acid, alanine and phenylalanine have been shown of their capability of getting covereted into carbohydrate. e.g. Aspartic acid ⟶ oxalacetic acid ⟶ Carbohydrate.

Alanine ⟶ Pyruvic acid ⟶ Carbohydrate.

According to the amino acids classification by Block, tryptophan and Phenylalanine are indispensable (essential) and aspartic acid and alanine dispensable amino acids. Also there is an increase in the rate of gluconeogenesis in diabetes. So, it was planned to study the effects of insulinase inhibitors, 1-tryptophan and niacin (nicotinic acid) indispensable amino acids phenyl-alanine (and 1-tryptophan); dispensable amino acids alanine and aspartic acid and auxins Indolyl-3-propionic and Indolyl-3-butyric acid on the potency of exogenous insulin both '*in vitro*' and *in vivo* in the normal fasting rabbits. (work was only on pilot scale as a preliminary for a large scale programme to be taken up at a future date).

Experimental

Preparation of amino acids solutions for 'in vivo' work

100 mg. of each acid was weighed and dissolved in 1 % Sod. Bicarbonate solution, pH was adjusted to 7-8 and volume made up to 5 ml.

Preparation of solution for 'in vitro' work

10 mg. of each acid was directly dissolved in one ml. of insulin solution (containing 2 units).

The injections and estimations of blood sugar were done in the same fashion as described in Part III.

Results

The values of blood sugar levels of fasting rabbits as effected by these substances are shown in the following tables. (No. [illegible])

TABLE X

Effects of 1-tryptophan on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

.....

No.	Time after injections	100 mg. of tryptophan & 2 units insulin (in vivo) (± S.E)	10 mg. of tryptophan and 2 units insulin (in vitro) (± S.E)
1.	0 (Fasting	94.82 mg. ±9.5	101.00 mg. ±9.6
2.	1 hr. 5 min.	73.70 mg. ±[illegible]	61.30 mg. ±4.6
3.	2 hours.	-	59.90 mg. ±[illegible]
4.	2 hrs. 5 min.	53.85 mg. ±4.3	-
5.	3 hrs. 5 min.	58.75 mg. ±5.1	-
6.	3 hrs. 15 min.	-	82.40 mg. ±7.8
7.	4 hours.	93.30 mg. ±8.1	-
8.	4 hrs. 15 min.	-	94.60 mg. ±8.1

TABLE XI

Effects of Nicotinic acid on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

.....

No.	Time after injections.	100 mg. of nicotinic acid and 2 units of insulin (*in vivo*)	10 mg. of nicotinic acid and 2 units of insulin (*in vitro*)
1.	0 (Fasting)	81.60 mg. ±7.6	90.00 mg. ± [illegible]
2.	1 hour.	65.64 mg. ± 5.5	45.95 mg. ± [illegible]
3.	2 hours.	52.57 mg. ± [illegible]	45.42 mg. ± [illegible]
4.	3 hours.	-	63.42 mg. ± 5.9
5.	3 hrs. 25 min.	58.90 mg. ±5.8	-

TABLE XII

Effects of phenylalanine on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

.....

No.	Time after injections.	100 mg. of phenylalanine and 2 units insulin (*in vivo*)	10 mg. of phenylalanine and 2 units of insulin (*in vitro*)
1.	0 (Fasting)	99.82 mg.	102.40 mg.
2.	1 hour.	86.63 mg.	54.20 mg.
3.	2 hrs. 15 min.	94.70 mg.	-
4.	2 hrs. 30 min.	-	50.56 mg.
5.	3 hours.	98.16 mg.	55.58 mg.

TABLE XIII

Effects of Alanine on the potency of insulin (2 units) in terms of glucose level/100 ml of blood of the normal fasting rabbits.

.....

No.	Time after injections	100 mg. of alanine and 2 units insulin (*in vivo*) [illegible]	10 mg. of alanine and 2 units insulin (*in vitro*) [illegible]
1.	0 (Fasting)	100.00 mg. ± 5.5	103.30 mg. ± 10.2
2.	55 minutes.	64.21 mg. ± 5.4	-
3.	1 hr. 55 min.	62.67 mg. ± 5.1	-
4.	2 hours.	-	47.40 mg. ± [illegible]
5.	3 hours.	-	54.81 mg. ± 5.1
6.	3 hours 5 min.	62.67 mg. ± 6.1	-
7.	4 hours.	-	64.26 mg. [illegible]

T A B L E XIV

Effects of aspartic acid on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

....

No.	Time after injections	100 mg. aspartic acid and 2 units insulin (*in vivo*)	10 mg. of aspartic acid and 2 units insulin (*in vitro*)
1.	0 (Fasting)	119.20 mg.	106.60 mg.
2.	1 hour.	72.53 mg.	97.56 mg.
3.	2 hours.	86.10 mg.	63.40 mg.
4.	3 hours.	87.50 mg. ±7.5	62.90 mg.

T A B L E XV

Effects of Indolyl-3-propionic acid on the potency of insulin (2 units) in terms of glucose level/100 ml. of blood of the normal fasting rabbits.

....

No.	Time after injections	100 mg. Indolyl-3-propionic acid & 2 units insulin (*in vivo*)	10 mg. Indolyl-3-propionic acid and 2 units insulin (*in vitro*)
1.	0 (Fasting)	112.20 mg.	100.00 mg.
2.	1 hour.	70.29 mg.	48.58 mg.
3.	2 hours.	84.40 mg.	54.09 mg.
4.	3 hours.	101.00 mg.	65.80 mg.

T A B L E XVI

Effects of Indolyl-3-butyric acid on the potency of insulin (2 units) in terms of glucose level/ 100 ml. of blood of the normal fasting rabbits

....

No.	Time after injections	100 mg. Indolyl-3-butyric acid and 2 units insulin (in vivo)	10 mg. of Indolyl-3-butyric acid and 2 units insulin (in vitro)
1.	0 (Fasting)	97.72 mg. ± 8.7	102.60 mg. ± 10.1
2.	1 hour.	48.10 mg. ± 4.1	54.16 mg. ± 4.6
3.	2 hours.	55.46 mg. ± 4.9	56.76 mg. ± 5.1
4.	3 hours.	67.00 mg. ± 6.1	63.00 mg. ± 5.6

Discussion

Table X reveals that the presence of 1-tryptophan along with insulin enhances ~~and prolongs~~ the action of insulin to some extent.

Table XI indicates the potentiating effects of nicotinic acid on insulin. Obviously the potentiation of insulin by nicotinic acid is much more as compared to that of tryptophan. This raises a fundamental question, nicotinic acid being a metabolite of tryptophan, whether the effect of tryptophan on insulin is due to its own action or is a secondary effect of its first metabolic conversion to nicotinic acid. Nicotinic acid being a member of vitamin B-complex forms propthetic groups of enzymes.

Table XII represents the effects of indispensable amino acid viz. phenyl-alanine. Whereas '<u>in vitro</u>' it enhances the potency of insulin to some extent, it does not show any increase in potency of insulin '<u>in vivo</u>' . It is possible that this acid may be forming some type of slowly diffusible complex with insulin, the type of which was reported (between insulin and sulphones) by Sahyun and Heyn (1940) though not confirmed by Macallum (1948). The'<u>in vivo</u>' effect could be possibly due to the reason that this acid being an essential amino acid, is possibly preferentially taken up for the purpose of some other

metabolic process and may not be available for the formation of this type of complex with insulin (as '*in vitro*').

Table XIII indicates the effects of alanine. It is clear that even though it is not an essential amino acid, it is intimately related in its structure to phenyl-alanine. Whereas its '*in vitro*' effect is like phenyl-alanine only, '*in vivo*' effect of this acid lies in prolonging the action of insulin. Probably the ease with which this acid could be changed into pyruvic acid and thereby enter the Krebs cycle, may be responsible for its '*in vivo*' effect. It has already been noted that the stimulation of Krebs' cycle by the increase of T.C.A. metabolites may enhance the glucose uptake and thereby cause a potentiation of insulin.

Inspection of Table XIV indicates a slight prolongation of insulin action by aspartic acid '*in vitro*', like that of alanine. Aspartic acid could enter the T.C.A. cycle by Metabolic deamination to oxaloacetic acid. The '*in vivo*' effect of course is almost as much as that of pure insulin.

Plant regulators or auxins Indolyl-3-propionic acid and Indolyl-3-butyric acid which have been suggested to be also insulinase inhibitors appear to have some effect on the insulin potency both '*in vitro*' and '*in vivo*' (Tables XV and XVI).

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PART V*

STUDIES ON THE EFFECTS OF METABOLITES ON THE BEHAVIOUR OF INSULIN ON THE CAPACITY OF DROPPING MERCURY ELECTRODE

*Results of this part have been partly published in Research Bulletin (N.S) Panjab University (1960); 11, 155.

A B S T R A C T

Behaviour of insulin on the capacity of dropping mercury electrode (DME) has been studied. Further, effects of a few metabolites on this behaviour have been investigated. It is noted that insulin behaves like a surfactant on the capacity of DME and is very little influenced by the presence of these metabolites.

I N T R O D U C T I O N

Levine *et al* (loc. cit) suggested the mode of action of insulin in terms of transfer of glucose across the cell memberane. This transport of glucose across the cell memberane stresses the importance of certain physical phenomena like permeability, surface activity, surface potential and the like.

There are three main hypotheses for the permeability phenomenon (Ruhland, 1955):

1) Mechanical .... the cell memberane is a sieve.

2) Chemical .... substances enter the cells by chemical combination or solubility.

3) Electrical .... substances enter the cells because of an electrical charge.

It is well known that protoplasmic surface is a dynamic system rather than merely a sieve. Overton (1900) noticed that non-polar molecules enter the cell more rapidly than the polar ones (like /OH, /COOH, etc.)

Further, the molecular weight of insulin as determined by osmotic pressure or ultra-centrifugal methods is 48000 and by X-ray measurements about 36000 (Sanger, 1956); whether a protein molecule of this size can penetrate the cell, if insulin acts intra cellularly, is a matter of conjecture.

A surface active substance can show adsorption and desorption under different conditions. These substances are capable of altering the capacity of a dropping mercury electrode.

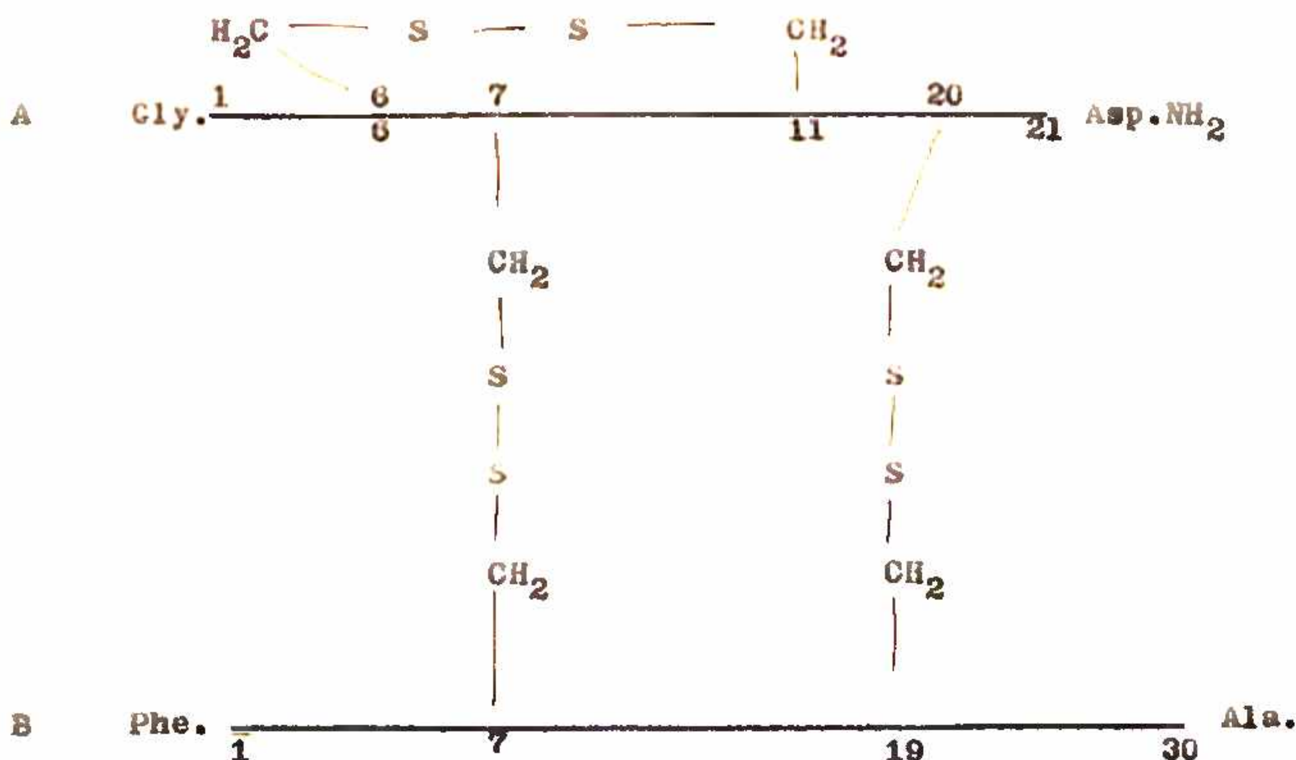
Doss and Kalyansundaram (1952) reported the effects of a good number of organic substances on the capacity of dropping mercury electrode at electro-capillary zero.

Randles (1947) reported that addition of gelatine in polarographic studies brought down the rate of discharge of cadmium ions by a thousand times. Keilin (1948) had also made a similar type of observation on the polarographic measurements. This effect has been attributed to the surface active substances getting adsorbed at the mercury surface and hindering electrode processes in the coveted areas. Thus, adsorbed surface active substances are known to cause a lowering in the capacity of the electrical double layer.

Frumkin and Proskurnin (1935) were the first to make a detailed study of the effects of surface active substances on the dropping mercury electrode. Due to the effects of surface active substances, there is a lowering of the capacity in the neighbourhood of the electro-capillary zero. This lowering of the capacity has been attributed to the adsorption of surface active substances which interfere with the electrode processes necessary for the passage of the capacitative current. A depression in the capacity in the neighbourhood of the electro-capillary zero has been observed in insulin by Gupta (1954) who observed that macromolecules are sluggish in adsorption and desorption in the pulsating field at the desorbing potentials. The desorption peak in the case of macromolecules, therefore, should be either non-existent or small.

The last ten years have seen the most remarkable advances ever recorded in the annals of protein chemistry. Sanger and his associates (1951, 1952, 1953 and 1954) have determined the structure of insulin. Harfenist's (1953) analysis of amino acids of insulin is in full accordance with the results of Sanger *et al*.

Insulin is a peptide and consists of two chains of 51 amino acid residues of 16 amino acids. The two chains (A & B) of the amino acids in insulin molecule are joined to each other by two covalent linkages of disulphide bridge. This structure could be shown as :



Crystalline insulin also contains small amounts of zinc (about one atom per 12,000 equivalent weight, which appears to be bound rather firmly. It has been suggested that insulin in blood circulates in a complex form with other substances particularly with large basic net charge (Antoniades et al 1956). This complex of insulin is thought to be a sort of a regulator for the equilibrium between "free" and "bound" insulin in blood. Free insulin is claimed to be the active form and in bound form insulin is biologically inactive for most of the tissues.

In view of the claim that insulin has something to do with the glucose transport across the cell membrane, it was

planned to study the effects of insulin on the capacity of dropping mercury electrode (D.M.E.) and further, to study any possible effects of metabolites on the effect of insulin on the capacity of D.M.E.

Experimental

1) Insulin solution was the medicinal product of Lilly and was of U.S.P specifications, potency of insulin being 40 units per ml.

2) Indifferent electrolyte used was 0.1 M KCl, pH adjusted at 4.00 with 0.1 N HCl in a Beckmann pH meter (Model H_2). To avoid any possible 'time effect' when the electrolyte was in contact with the pool of mercury, 0.1 M KCl solution was stored in contact with a little of mercury and calomel.

3) Metabolites studied were: glucose, Sod. beta-hydroxy-butyrate, Sodium Isocitrate, Sodium Citrate and Sodium Succinate.

8.0 mg. of each of these metabolites were used in each experiment.

4) D.C. potentials throughout this study have been expressed with reference to the saturated calomel electrode.

The constants of mercury were:

t = 1.79 sec. per drop, open circuit in 0.1 M KCl.

5) The set up of dropping mercury electrode was similar to the one described by Doss and Gupta (1952).

The technique consists of applying to D.M.E. A.C. ripples (50 cycles) of ± 45 mV (r.m.s), over the D.C. potential and observing the alternating component. As impedance of the dropping mercury electrode is much higher than the rest of the impedence of the system, the magnitude of the alternating current gives a measure of the capacity of D.M.E.

A vacuum tube voltmeter was used as an amplifier and the amplified current was rectified and measured by a galvanometer with a suitable shunt. Since the resistive impedence due to the rest of the circuit was low as compared to the impedence due to the capacity of the DME , the galvanometer deflections were nearly proportional to the differential capacity (circuit diagram is given in Fig. 1.)

Figure 1.

Procedure

For control readings, 9 ml. of 0.1 M KCl (pH 4.0) were taken and by varying the D.C. potential, the A.C. impulse of the resistance applied to bring back the galvanometer reading to the same point on the lamp and scale arrangement, was noted.

FIG. 1

CIRCUIT DIAGRAM OF DROPPING MERCURY ELECTRODE

VARIABLE RESISTANCE BOX

Hg POOL

R_1 = 7500 Ω

R_2 = 11000 Ω

R_3 = 55 Ω

R_4 = 3.5 Ω

For the study of insulin, 8.0 ml. of indifferent electrolyte along with 1.0 ml. of 40 units per ml. crystalline zinc insulin (Lilly) were used.

For the study of the effects of metabolites to 8.0 ml. of indifferent electrolyte containing 8.0 mg. of the metabolite, 1.00 ml. of insulin was added. Readings were taken in the usual way.

Results

The effect of indifferent electrolyte, 0.1 M KCl on the capacitance of D.M.E. is given in Table XVII.

Effects of 1 ml. insulin on the capacitance are given in Table XVIII.

Effects of Glucose are given in Table XIX.

Effects of Sod. beta-hydroxybutyrate are given in Table XX.

Effects of Sod. isocitrate are given in Table XXI.

Effects of Sod. citrate are given in Table XXII.

Effects of Sod. succinate are given in Table XXIII.

TABLE XVII

Effect of 9 ml. of 0.1 M, KCl (pH 4.0) on the capacitance of dropping mercury electrode

.....

No.	D.C. applied in volts (corrected in terms of saturated calomel electrode).	A.C. in terms of ohms.
1.	0.0342	131
2.	0.1342	210
3.	0.2342	198
4.	0.3342	190
5.	0.4342	211
6.	0.5342	278
7.	0.6342	342
8.	0.7342	389
9.	0.8342	425
10.	0.9342	456
11.	1.0342	466
12.	1.1342	467
13.	1.2342	539
14.	1.3342	549
15.	1.4342	466
16.	1.5342	574
17.	1.6342	575
18.	1.7342	570

$$\%\ \text{increase of current (with origin)} = \frac{(\text{Initial current} - \text{Final current}) \times 100}{\text{Final current}}$$

T A B L E XVIII

Effects of 1 ml. insulin on the capacitance of dropping mercury electrode with 0.1 M KCl (8 ml. pH 4.00)

......

No.	D.C. applied in volts (corrected interms of saturated calomel electrode).	A.C. in terms of ohms.	% increase of A.C.
1.	0.0342	103	+ 27.18
2.	0.1342	170	+ 23.52
3.	0.2342	239	- 13.01
4.	0.3342	358	- 46.65
5.	0.4342	490	- 56.73
6.	0.5342	590	- 51.18
7.	0.7342	729	- 46.67
8.	0.8342	720	- 40.97
9.	0.9342	696	- 34.48
10.	1.0342	629	- 25.91
11.	1.1342	580	- 19.48
12.	1.2342	670	- 19.55
13.	1.3342	639	- 14.08
14.	1.4342	620	- 8.70
15.	1.5342	602	- 4.65
16.	1.6342	545	+ 5.50
17.	1.7342	519	+ 9.82

TABLE XIX

Effects of Glucose (8 mg) on the effects of insulin 1 ml. with 8 ml. of 0.1 M KCl(pH 4.0) on the behaviour of dropping mercury electrode.

....

No.	D.C. applied in volts (corrected in terms of saturated calomel electrode).	A.C. in terms of ohms.	% increase of A.C.
1.	0.0342	105	+ 24.76
2.	0.1342	174	+ 26.89
3.	0.2342	243	- 18.51
4.	0.3342	330	- 42.42
5.	0.6342	689	- 50.36
6.	0.7342	799	- 51.32
7.	0.8342	795	- 46.55
8.	0.9342	738	- 38.21
9.	1.0342	652	- 29.90
10.	1.1342	565	- 17.52
11.	1.2342	609	- 11.49
12.	1.3342	580	- 5.34
13.	1.4342	570	- 0. 70
14.	1.5342	547	+ 4.93
15.	1.6342	488	+ 18.03
16.	1.7342	498	+ 14.44

TABLE XX

Effects of Sod. beta-hydroxybutyrate (8 mg) on the effect of 1 ml. insulin with 8 ml. of 0.1 M KCl (pH 4.0) on the behaviour of dropping mercury electrode.

....

No.	D.C. applied in volts (corrected in terms of saturated calomel electrode).	A.C. in terms of ohms.	% increase of A.C.
1.	0.0342	112	+ 17.00
2.	0.1342	172	+ 22.09
3.	0.2342	242	- 23.14
4.	0.3342	328	- 42.07
5.	0.4342	393	- 46.31
6.	0.5342	452	- 38.49
7.	0.6342	565	- 39.47
8.	0.7342	610	- 34.59
9.	0.8342	615	- 30.89
10.	0.9342	623	- 26.80
11.	1.0342	574	- 18.81
12.	1.1342	563	- 17.05
13.	1.2342	579	- 6.90
14.	1.3342	553	- 0.72
15.	1.4342	558	+ 1.43
16.	1.5342	559	+ 2.68
17.	1.6342	520	+ 10.57
18.	1.7342	535	+ 0.87

TABLE XXI

Effects of Sod. isocitrate (8 mg) on the effects of 1 ml. insulin with 8 ml. 0.1 M KCl(pH 4.0) on the behaviour of dropping mercury electrode.

......

No.	D.C. applied in volts (corrected in terms of saturated calomel electrode).	A.C. in terms of ohms.	% increase of A.C.
1.	0.0342	98	+ 34.79
2.	0.1342	170	+ 23.52
3.	0.2342	255	- 22.35
4.	0.3342	348	- 45.40
5.	0.4342	433	- 51.27
6.	0.5342	453	- 38.62
7.	0.6342	543	- 36.64
8.	0.7342	579	- 33.19
9.	0.8342	593	- 28.33
10.	0.9342	593	- 21.44
11.	1.0342	557	- 16.33
12.	1.1342	514	- 9.12
13.	1.2342	550	- 2.00
14.	1.3342	526	+ 4.37
15.	1.4342	552	+ 2.47
16.	1.5342	559	+ 2.66
17.	1.6342	549	+ 4.71
18.	1.7342	546	+ 4.39

T A B L E XXII

Effects of Sod. citrate (8 mg) on the effects of insulin 1 ml. and 8 ml. 0.1 M KCl (pH 4.0) on the behaviour of dropping mercury electrode.

......

No.	D.C. applied in volts (corrected in terms of saturated calomel electrode).	A.C. in terms of ohms.	% increase in A.C.
1.	0.0342	106	+ 23.58
2.	0.1342	174	+ 20.69
3.	0.2342	264	- 21.96
4.	0.3342	370	- 48.64
5.	0.4342	467	- 54.81
6.	0.5342	490	- 43.26
7.	0.6342	530	- 35.46
8.	0.7342	559	- 30.41
9.	0.8342	587	- 27.59
10.	0.9342	608	- 25.00
11.	1.0342	590	- 21.00
12.	1.1342	520	- 10.19
13.	1.2342	560	- 3.89
14.	1.3342	537	+ 2.23
15.	1.4342	545	+ 3.85
16.	1.5342	547	+ 4.93
17.	1.6342	535	+ 7.47
18.	1.7342	530	+ 7.54

TABLE XXIII

Effects of Sod. succinate (8 mg) on the effects of 1 ml. insulin with 8 ml. 0.1 M KCl(pH 4.0) on the behaviour of dropping mercury electrode.

......

No.	D.C. applied in volts (corrected in terms of saturated calomel electrode).	A.C. in terms of ohms.	% increase in A.C.
1.	0.0342	109	+ 20.18
2.	0.1342	189	+ 11.11
3.	0.2342	250	- 20.80
4.	0.3342	338	- 44.11
5.	0.4342	408	- 45.28
6.	0.5342	462	- 39.81
7.	0.7342	625	- 37.76
8.	0.8342	628	- 32.32
9.	0.9342	620	- 26.45
10.	1.0342	582	- 19.93
11.	1.1342	560	- 15.09
12.	1.2342	563	- 4.26
13.	1.3342	550	- 0.18
14.	1.4342	559	+ 1.07
15.	1.5342	562	+ 2.13
16.	1.6342	570	+ 0.87
17.	1. 1.7342	573	+ 0.52

All these results have been graphically shown in Fig. 2 to 7.

Discussion

There are a number of factors which play a part in the adsorption of substances at the mercury-water interface. In the case of surface active substances, such as proteins, the main factors causing adsorption appear to be the "squeezing type" of forces. Liquid water consists of a net work of hydrogen bonds. The hydrophobic groups like hydrocarbon and sulphide, in the molecule do not fit into this net work. In view of this, there is a tendency for the hydrophobic groups to be thrown out of the liquid. This tendency has been called by Miller and Grahame (1957) as the "Squeezing type". In this connection, these authors observed that :

(a) even short chain hydrocarbons are hydrophobic and produce a squeezing out effect which appears as a desorption anion and

(b) polybasic anions are completely expelled from the interface when the mercury carries even a small negative charge and strongly attracted when the charge is slightly positive. Adsorption and desorption peaks are produced at the potential of electro-capillary maximum (e.c. max.) through the operation of this effect. It seems that a monolayer of anions is formed.

Adsorption on mercury can take place in four different ways:

(a) Type I Adsorption: On an uncharged surface.

(b) Type II Chemisorption: Many anions are capable of forming bonds with mercury and the strength of these bonds depends upon the surface charge density. These anions ($''S$, $'I$, $'SCN$, $'Br$ etc.) are those which form insoluble salts with mercury. One can say that either a covalent bond is formed or can attribute the attraction to the polarizability of the anion. Type II adsorption is recognisable at e.c. max. when the charge on the mercury drops is zero. The adsorption of the anions lowers the surface tension and shifts the potential of e.c. max. towards more cathodic values.

(c) Type III Adsorption exhibited by anions like $'NO_3$, $'ClO_4$ and $'ClO_3$ etc. which are adsorbed at e.c. max. but obviously cannot form covalent linkages.

(d) Type IV is expected when one stops to consider the possibility of the lateral interaction between ions which have been "squeezed" out of the solution. Monolayers of unionized molecules are formed at the interface and the vander Waal forces help to stabilize them.

The operation of these 'squeezing type of forces' manifest themselves in a number of well-known phenomena, such as

1) Insolubility of hydrocarbons in water.

2) insolubility of substances having molecules with a preponderant proportion of hydrophobic groups;

3) adsorption of surface active substances such as soaps, dye-stuffs, proteins etc. at (a) water-air interface causing foam stability, (b) water-oil interface causing emulsion stability, (c) water-carbon interface affecting wettability, (d) water-metal interface causing the phenomena of protection and sensitization ;

4) micelle formation in soaps, dye-stuffs and the other surface active substances at higher concentrations ;

5) complex formation between the indicators and the surface active substances;

6) adsorption of wetting agents on the wax covered cellulose capillaries in yarn, thereby causing the wetting phenomena and

7) orientation during the spreading of surfactants.

Insulin molecule as shown from its structure has got a number of hydrophobic groups. Insulin molecules, therefore, tend to spread on water surfaces. It is of great interest to note that insulin molecule loses its biological activity if the hydrophobic group like the disulphide bridge is destroyed. Insulin isolated from different animal species has got this vital structural unit viz. the disulphide bridge in every species.

The adsorption of surface active substances like insulin takes place unhindered at the mercury water interface at

the potential corresponding to e.c.max. and this potential represents the stage at which the surface has no charge. The value of this potential as found in this study is about - 0.3 V with reference to the normal calomel electrode. The adsorption therefore, would be maximum in the neighbourhood of this potential.

If the potential of D.M.E. is made highly negative or positive as compared to electrocapillary zero, the interface gets a high charge. This causes strong attractive forces between the charged surface and the water dipoles. In case the surface is negatively charged, it tends to pick up the water dipoles by the positive ends and if positively charged, it tends to pick up water dipoles by the negative ends. In either case, the attraction is so strong that the 'squeezing type' of forces are not able to compete with these forces; so much so the surfactants get desorbed. In the present study with insulin this desorption took place at the potential of - 1.6 V, pH being 4.0 (Fig. 2). This behaviour would be influenced by the net charge of the adsorbed molecules.

If a complex formation is possible between insulin and metabolites, such a complex formation would affect the adsorbility of insulin. So, it becomes all the more important to examine the effect of glucose on the adsorption behaviour of insulin. A comparison of Fig. 2 (for insulin) and Fig. 3 (for glucose and insulin) shows that this type of effect is not

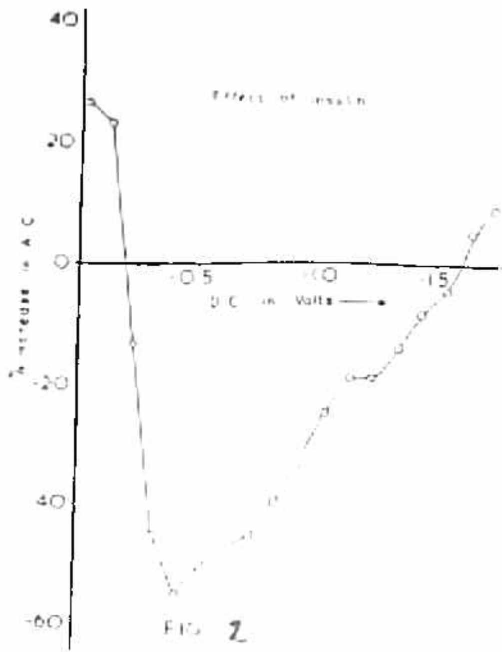


FIG 2

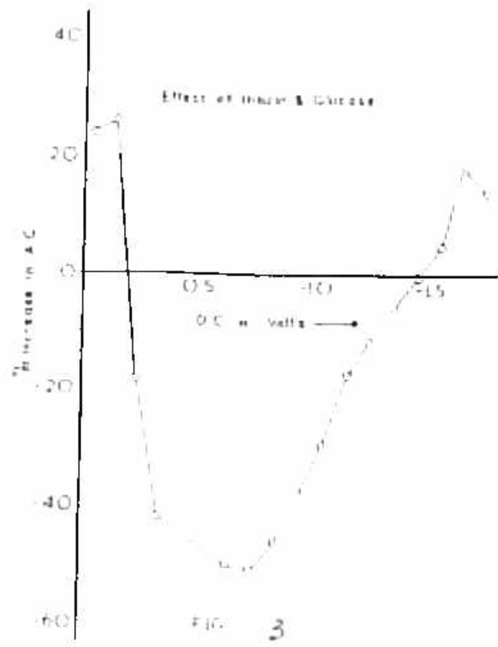
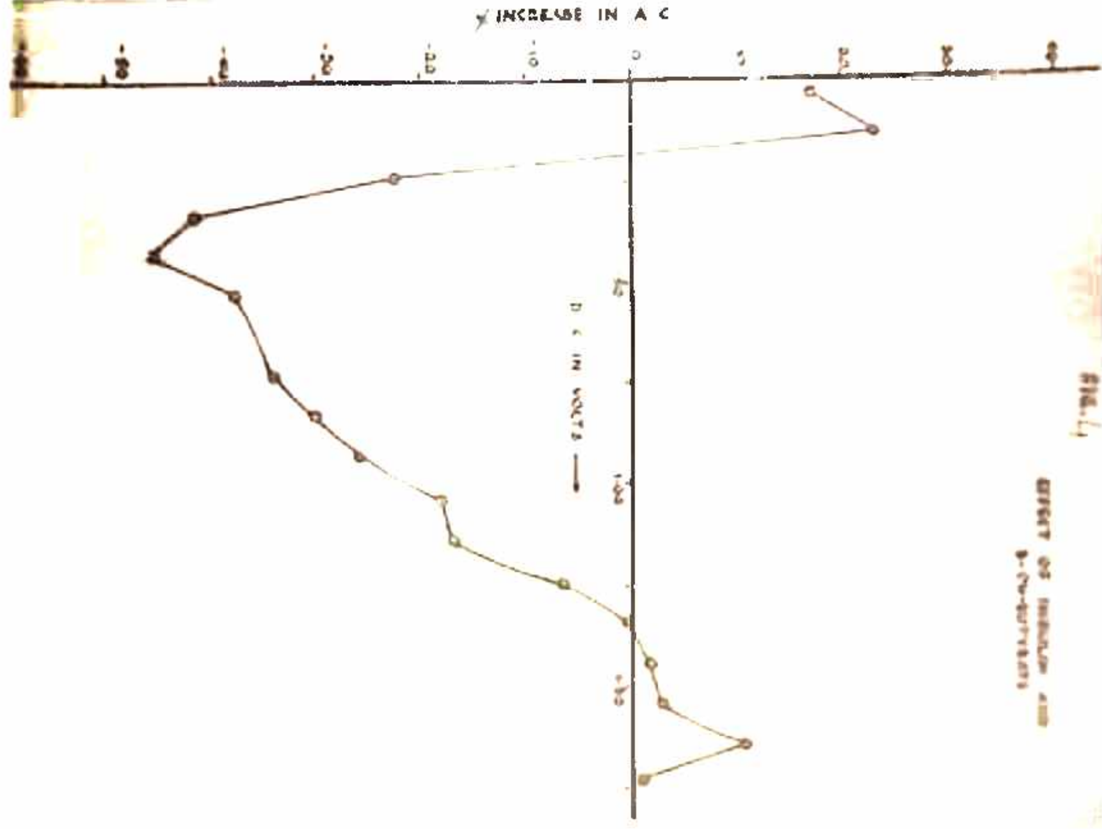


FIG 3

much, from which it is concluded that as studied by this method the complex formation of glucose and insulin is possible to a very little extent only.

A comparison of the Figs. 2 & 4 shows, however, that there is a considerable difference in the behaviour between pure insulin and insulin mixed with Sod. beta-hydroxybutyrate. The later system shows a lowered adsorption as well as lowered voltage for desorption. Whereas the maximum depression on capacity is 56 % with insulin, it is only about 46 % with this mixture. This indicates that the adsorbility of the complex is less than that of insulin. This gives rise to the prominence of an interesting conclusion whether in diabetes, the rise in concentration of ketone bodies like beta-hydroxybutyrate can influence the mode of action of insulin. In case such type of insulin-beta-hydroxybutyrate complex could be possible in a biological system, it would influence the activity of insulin in bringing about the cells in more intimate contact with the substrates.

A comparison of Fig. 2 with Figs. 5,6 & 7 shows that sodium isocitrate, sodium citrate and sodium succinate are also capable of influencing to some extent the behaviour of insulin on DME. It is evident from these figures that the desorption peak as formed in the case of beta-hydroxybutyrate is not observed in the case of these T.C.A. members which suggests the possibility of some different type of effect on


% INCREASE IN A C
D C IN VOLTS

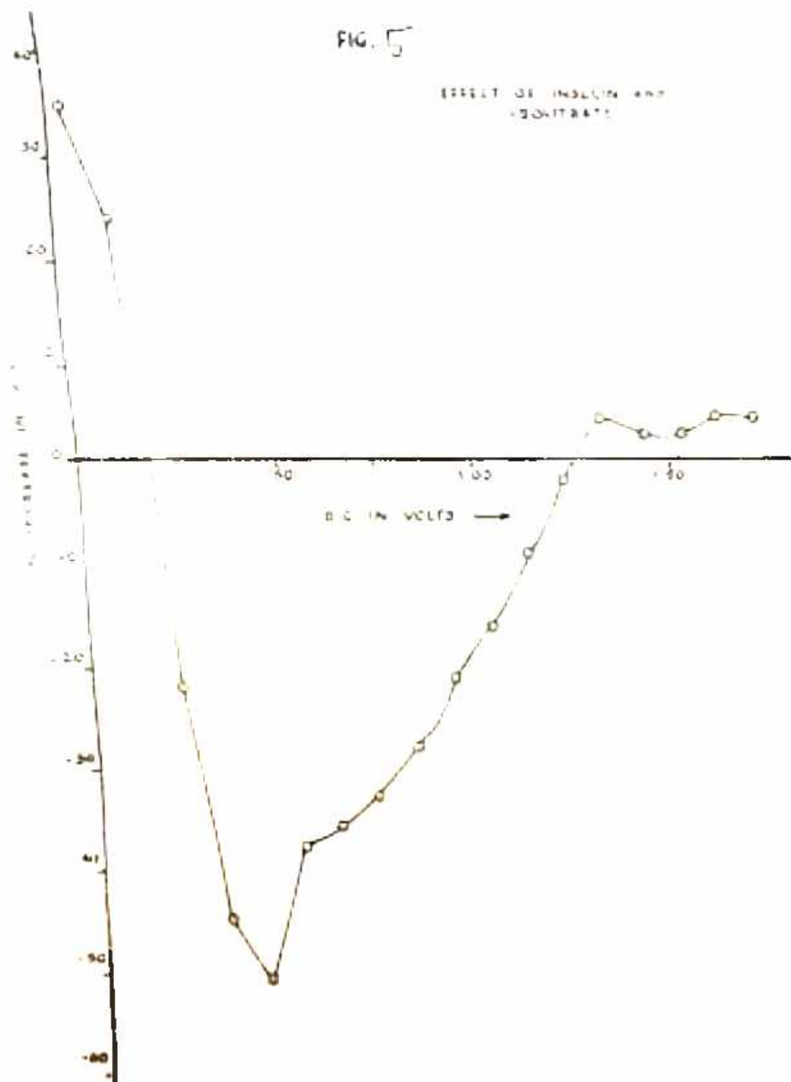


FIG. 5
D.C. IN VOLTS

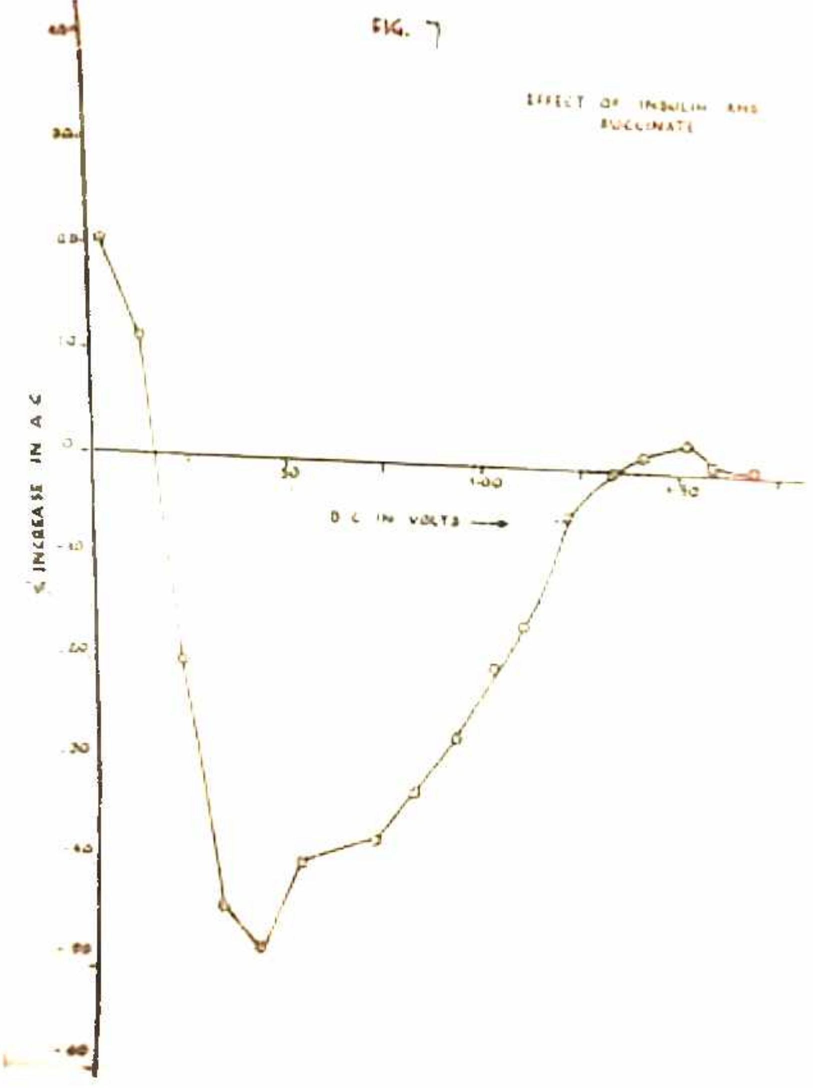
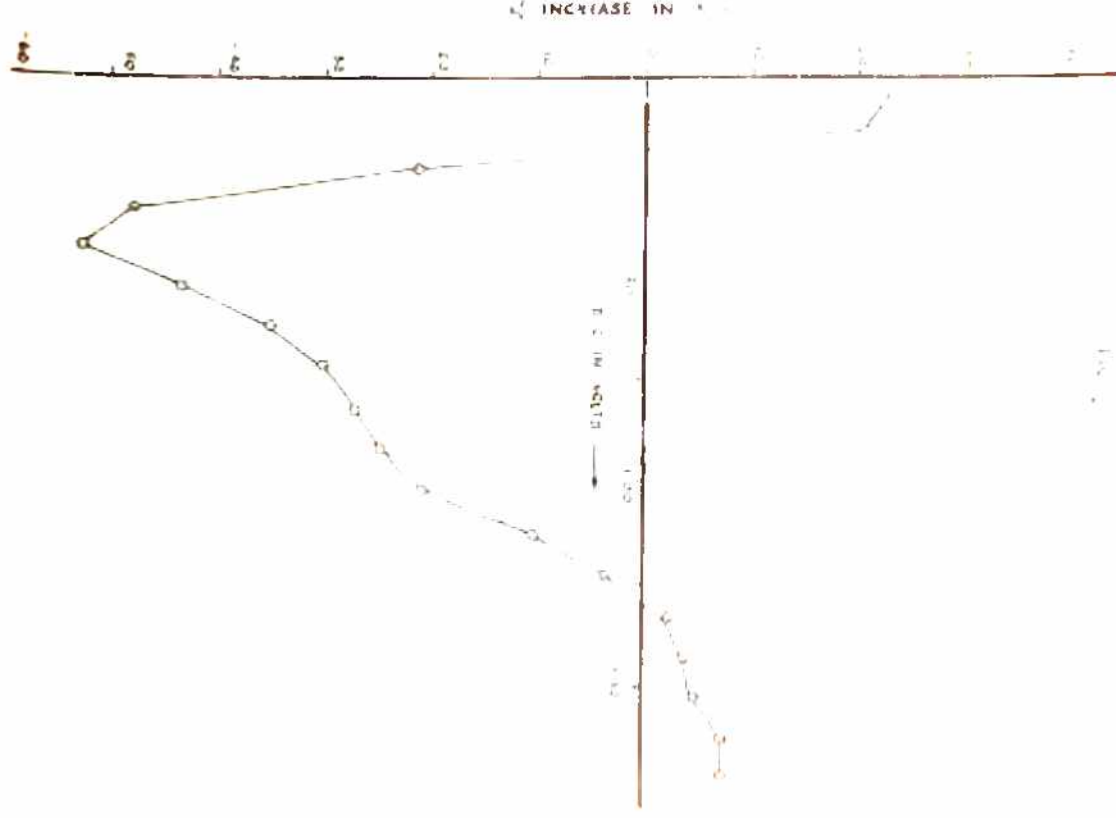


FIG. 7

EFFECT OF INSULIN AND SUCCINATE



INCREASE IN

insulin.

The occurrence of adsorption by way of decrease in the double layer capacity is shown in this study. The magnitude of alternating current in A.C. polarographic curves is a measure of this adsorption.

To explain the formation of desorption peak, there are some recent observations. The peak capacity at desorption potential attains a value which can be very much higher than the capacity with pure electrolyte at that potential. The enormous peaks observed in the differential capacity have been explained as produced by the excursions of the potential due to the superimposed A.C. ripple causing sorption and desorption process, which are supposed to cause a high dynamic capacitance. Recently, Doss and Venkatesan (1959) have observed that there is no difference in the capacity at the desorption potential of pure indifferent electrolyte which was stirred and the surface active substance under stirred conditions. They concluded that surface active substances may be causing some sort of directed movement of the liquid near the mercury drop. This they actually observed to be so by a simple optical arrangement. These authors on the basis of their findings conclude that the polarographic maxima as well as the desorption peaks in tensammetric curves are caused by electrocapillaryphoretic movements mainly due to the effect of fresh surface emerging from the capillary tip. The non-occurrence of desorption peaks in the case of insulin, as noticed in the present study, might be caused by the

relative immobility of the interfacial layer when it is having surface active substances of high molecular weight. Gupta (loc. cit.) also proposed the sluggishness of macromolecules for sorption and desorption.

If lowered values of hydrophobic groups could lower down the "Squeezing type of forces" in biological systems too, observations of Ralli et al (1955) regarding the lowered concentration of sulphahydryl groups in diabetes would be of significance, thereby, suggesting a derangement in the role of insulin.

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PART VI

STUDIES ON THE EFFECTS OF METABOLITES ON THE CHROMATOGRAPHIC BEHAVIOUR OF INSULIN

ABSTRACT

Effects of a few metabolites on the chromatographic behaviour of insulin have been studied. Results obtained have been discussed in the light of difficulties involved in the chromatographic study of insulin.

INTRODUCTION

The biological methods of insulin assay are much cumbersome and costly. There had been attempts to replace biological methods by simpler and more convenient '*in vitro*' methods.

The need of a non-biological assay method is all the more necessary when many samples containing micro-quantities of insulin are to be estimated.

The fibril-precipitation method was devised by Waugh *et al* (1950) and this method was further modified by Grodsky (1958). Foster *et al* (1951) reported the failure of fibril method in the case of low potency insulin solution.

Attempts of devising *in vitro* assay methods of insulin had been considered to be worthwhile by adapting chromatographic techniques. Porter (1953), Porath and Li (1954),

Dickinson (1956), Robinson and Fehr (1952), Grodsky and Traver (1956), Light and Simpson (1956), all devised chromatographic methods for insulin assay by making use of column or paper chromatographic techniques.

Light and Simpson (1956) found that the biological activity of insulin is associated with a specific spot on the paper chromatogram and that the staining dye bound by this spot under specific conditions is in linear relationship with the concentration of biologically active insulin. Keeping these views as the basis, Fenton (1959) devised a method for the estimation of insulin potency by paper chromatography.

Such a method could be of great use in determining directly the effect of any substance on the potency of insulin. In this method any non-proteinous substance would not interfere with insulin assay as the staining agent would specifically stain only peptides or proteins. So, it was planned to study the effects of metabolites on the insulin potency by chromatographic method by following the method of Fenton (1959) with the modification that instead of ascending, descending chromatography was made use of.

Experimental

(a) Insulin used was crystalline zinc insulin (Lilly) U.S.P. with a potency of 40 units per ml.

(b) Substances which were used to study their effects on insulin potency were :

1) Glucose (B.D.H.)
2) DL-Sod. beta-hydroxybutyrate (Light & Co.)
3) Sod. Isocitrate (Nutrition & Biochem)
4) Sod. Citrate (E. Merck)
5) Sod. Succinate (E. Merck)

(c) Preparation of solution:

1) 0.5 ml. insulin U.S.P. (Lilly) was directly taken into each of a number of Pyrex tubes with glass stoppers.

2) 5 mg. of a metabolite (listed above) was added to one tube and the tube shaken so as to dissolve the substance.

0.1 ml. of this insulin metabolite solution so formed was applied on the starting line of a Whatman Filter Paper No. 1 (Chromatographic grade). The size of the paper was (35 cm. long x 22 cm. wide) and the spots were applied at distances of 3 - 4 cm. from each other. These spots were partly dried by blowing air at room temperature with the help of an electric hair drier. The filter papers were left suspended in the draught free room for 6 - 7 hours so that the insulin spots were dry.

(d) Developing solvent: Butan-2-01 (E. Merck chromatographic grade) and 1 % acetic acid (B.D.H.) were mixed in the ratio of 1 : 1 (v/v), shaken vigorously for several minutes in a Pyrex separating funnel. This mixture was left in a separating funnel for 24 hours after which the lower aqueous phase was discarded.

(e) Staining solution: Acetic acid (B.D.H.) 12 ml. was diluted to 2 litres and 125 ml. of 0.1 N NaOH was added to it. 0.4 g. of Bromocresol green (B.D.H.) was dissolved in this solution. The pH of this solution was 3.6. This staining fluid was prepared every fortnight.

(f) Eluent: Equal parts of 0.1 N NaOH and 95 % aldehyde free ethanol were mixed just before elution.

(g) Assay: The paper on which the spots of insulin were applied was fixed up in the descending chromatographic glass chamber and was brought to equilibrium with the vapours of developing solution, a small quantity of which was kept in a beaker at the bottom of the chamber. The paper was left overnight in this position for attaining equilibrium, and the assay was done at room temperature. The chromatographic chamber was covered with a glass plate with the help of silicone grease.

Next day, the solvent was added and sufficient time was given for the solvent front to travel to a convenient distance.

The paper was removed from the chamber and dried by hot air current and further kept in an oven at 80°C for 10 minutes. After that the paper was immersed in the staining solution for 15 hours. Paper was washed in 1 % (v/v) acetic acid for 3 minutes three times and then pressed in blotting paper sheets, further dried in warm air and returned to an oven at 80°C for 10 minutes. The colour of the insulin stains was further intensified by exposure to the vapours of ammonia.

(h) Determination of RF values: It was determined by measuring the distance travelled by the solvent front from the starting line and the distance of the centre of the stained insulin spot from the centre of the insulin spot applied.

$$\text{RF value} = \frac{\text{Distance of the stain from the spot applied}}{\text{Distance travelled by the solvent front.}}$$

(i) Elution and determination of % of transmission: The spots were outlined with pencil so as to cover equal areas, cut out and placed in different tubes. To each tube 5 ml. of the eluent was added and the tubes agitated at intervals for 30 minutes and then percentage of transmission of the standard insulin was compared with that of insulin with metabolites in a spectrophotometer (Hilger U.V. Spek) at 625 mμ in 10 mm cells. For elution the entire protein stains spots were eluted.

Each experiment of chromatographic assay had its own standard insulin spots which were always the outer-most on the corners of the filter paper.

R e s u l t s

Effects of these metabolites on the RF value of insulin are given in Table XXIV.

Table XXV gives the RF values of insulin as affected by the rest of the metabolites; in this case the solvent front had moved slightly less than the distance travelled by the solvent front in values given in Table XXIV.

Tables XXVI and XXVII show the effects of these metabolites on the percentage transmission of insulin.

TABLE XXIV

Effect of different metabolites
on RF value of insulin.

.....

No.	Metabolite added.	RF value (average of 2 readings).
1.	- (Insulin only)	0.38
2.	Sod. betahydroxybutyrate trace in 4 units insulin	0.33
3.	Glucose 5 mg. in 4 units insulin.	0.28

TABLE XXV

Effect of the rest of metabolites
on the RF value of insulin

.....

No.	Metabolite added(5 mg. in 4 units insulin)	RF value (average of 2 readings).
1.	- (insulin only)	0.35
2.	Sod. Isocitrate.	0.26
3.	Sod. citrate.	0.25
4.	Sod. Succinate.	0.28

TABLE XXVI

Effect of metabolites on the colour Development of insulin spots.

......

No.	Metabolite added	Percentage transmission	Optical Density.
1.	- (insulin only)	30.6	0.515
2.	Sod. beta-hydroxy-butyrate.	23.6	0.630
3.	Glucose	21.9	0.660

TABLE XXVII

Effect of metabolites on the colour development of insulin spots

.....

No.	Metabolite added.	Percentage transmission.	Optical Density.
1.	- (insulin only)	35.5	0.450
2.	Sod. Isocitrate.	36.1	0.442
3.	Sod. Citrate.	33.7	0.464
4.	Sod. Succinate	33.8	0.470

Discussion

It is noted that the RF value of insulin is directly proportional to the distance moved by the solvent front. Similar observations have also been made by Light & Simpson (1956) and Fenton (1959).

Lowering of RF values of insulin in the presence of metabolite indicates that in some way the mobility of insulin could be affected by these metabolites.

The staining technique is non-specific as it stains all types of proteins and so, in the present study though the percentage transmission of insulin is affected by the metabolites it is difficult to say on the basis of chromatographic study only that the potency of insulin is also affected.

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STUDIES ON THE EFFECTS OF SOME METABOLITES ON THE BEHAVIOUR OF INSULIN ON THE CAPACITY OF DROPPING MERCURY ELECTRODE

I. Effect of Glucose

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Abstract

Behaviour of insulin on the capacity of dropping mercury electrode has been studied. Further effect of glucose on this behaviour has also been studied. A possible explanation of this behaviour has been put forward.

Introduction

Levine and others (1950) on the basis of experimental data suggested that the mode of action of insulin may be explained in terms of glucose transport across the cell membrane. Insulin is a macromolecule with high molecular weight. How, and whether a peptide molecule of this size could penetrate the cell membrane within which it appears to perform its unique function, is a matter of conjecture. It is quite pertinent, therefore, to think of some physico-chemical phenomena which play an important role in the mode of action of insulin.

Since insulin has got a good number of hydrophobic groups, one should expect such a molecule to possess spreading power and surface activity. Surface active substances show adsorption and desorption under different conditions. These substances can alter the capacity of dropping mercury electrode (D.M.E.). Frumkin and Proskurnin (1935) were the first to make a detailed study of the effects of surface active substances on D.M.E. Doss and Kalyansundram (1952) have reported the effects of a good number of organic substances on the capacity of D.M.E. Depression of the capacity in the neighbourhood of the electrocapillary zero has been observed in insulin by Gupta (1954). But there is no report so far on the effects of medicinal insulin and how this behaviour could be affected by different metabolites. Preliminary observations on the effects of medicinal insulin on the capacity of D.M.E. have been reported in this communication and further, attempts have been made to detect the possibilty of any glucose-insulin complex formation by this method.

Material and Methods

(*i*) Insulin solution was zinc crystalline medicinal type product of Lilly (E. I. Lilly & Co., Rochester, U.S.A.) and was of U.S.P. specifications; potency of insulin being 40 units per ml.

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