STUDIES ON THE MEASUREMENT OF SERUM INSULIN ACTIVITY

by

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The evidence in the current literature suggests that insulin acts by increasing the rate of transfer of glucose into the cell.

A reliable method for the estimation of serum insulin activity would be of value in helping to elucidate the physiological role of insulin, and the etiology and pathogenesis of diabetes mellitus and other disorders of carbohydrate metabolism.

The in vivo assay method measures the serum insulin activity by the drop in blood sugar produced in alloxan-diabetic hypophysectomized adrenalectomized mice. The reliability of this method has not been satisfactorily proven and, in addition, the animals are unstable and difficult to maintain.

The in vitro assay method measures the serum insulin activity by the rate of glucose uptake of the isolated rat diaphragm. This method and the in vivo method cited above, measure the net serum insulin activity. One modification of the in vitro method was investigated and found to give extremely inconsistent results in our hands.

A method for the estimation of absolute serum insulin activity was devised. This method compares the glucose uptake of a rat hemi-diaphragm immersed in serum with that of the corresponding hemi-diaphragm immersed in serum whose insulin has been inactivated by the addition of cysteine. The serum insulin activities of twelve normal fasting adults were measured. It was found that these values were enhanced by the ingestion of glucose or by the injection of insulin. A diabetic deprived of insulin was found to have no serum insulin activity while an infant with idiopathic spontaneous hypoglycemia was found to have an elevated serum insulin activity. The in vitro addition of insulin to serum was found to enhance the insulin activity of that serum. The threshold for the production of this effect was 75 milli-units per ml.

TABLE OF CONTENTS

Chapter		Page
I	The Mechanism of Insulin Action	1
	A. General Actions	1
	B. Carbohydrate Metabolism	1
	C. Site of Action	3
	1. The Transfer Mechanism	4
	2. The Hexokinase Reaction	6
	D. Summary	8
II	Current Methods of Insulin Assay	10
	A. Introduction	10
	1. Biological Assay in Vivo	10
	2. Biological Assay in Vitro	11
	3. Assay by Antigen-Antibody Reaction	14
	B. Experimental	14
	1. Methods	14
	2. Results	15
	C. Discussion	17
	D. Conclusions	19
III	The Measurement of Absolute Serum Insulin Activity	21
	A. Introduction	21
	B. Method	21
	C. Results	22
	1. Normal Fasting Serum Insulin Activity	22
	2. The Effect of in Vitro Addition of Insulin	24
	3. The Effect of Ingestion of Glucose	24
	4. The Effect of Intra-Venous Injection of Insulin	24
	5. Idiopathic Spontaneous Hypoglycemia of Infancy	28
	6. Serum Insulin of a Diabetic After Withdrawal of Insulin	28
	D. Discussion	28
	E. Summary and Conclusions	30

CHAPTER I. THE MECHANISM OF INSULIN ACTION

GENERAL ACTIONS:

Insulin, the hormone produced by the beta cells of the pancreatic islets, has been observed to affect the metabolism not only of carbohydrate but also of fat and protein. The changes it leads to include lowering of blood sugar, stimulation of muscle glucose utilization and glycogen synthesis, stimulation of liver glycogen synthesis, stimulation of fat synthesis from acetate, inhibition of ketone body production and the inhibition of gluconeogenesis from protein. In investigating the mechanism of action of insulin, workers have sought to find a single site of action in the metabolic scheme which would explain all the observed effects of this hormone.

CARBOHYDRATE METABOLISM:

In order to understand the proposed theories of insulin action, it is necessary first to examine the principal reactions involved in glucose metabolism and their relation to the metabolism of fat and protein. These reactions are illustrated diagramatically in Fig. 1. From this diagram it is seen that the initial step in the metabolism of glucose is its reaction with adenosine triphosphate to form glucose-6-phosphate. This reaction is catalyzed by the enzyme gluco-hexokinase and is not reversible. The glucose-6-phosphate so formed can be used to synthesize glycogen, or can enter the glycolytic cycle in which series of reactions one mol of the six carbon compound is converted to two mols of a three carbon compound. One anerobic oxidation step takes place and the result of anerobic glycolysis of one mol of

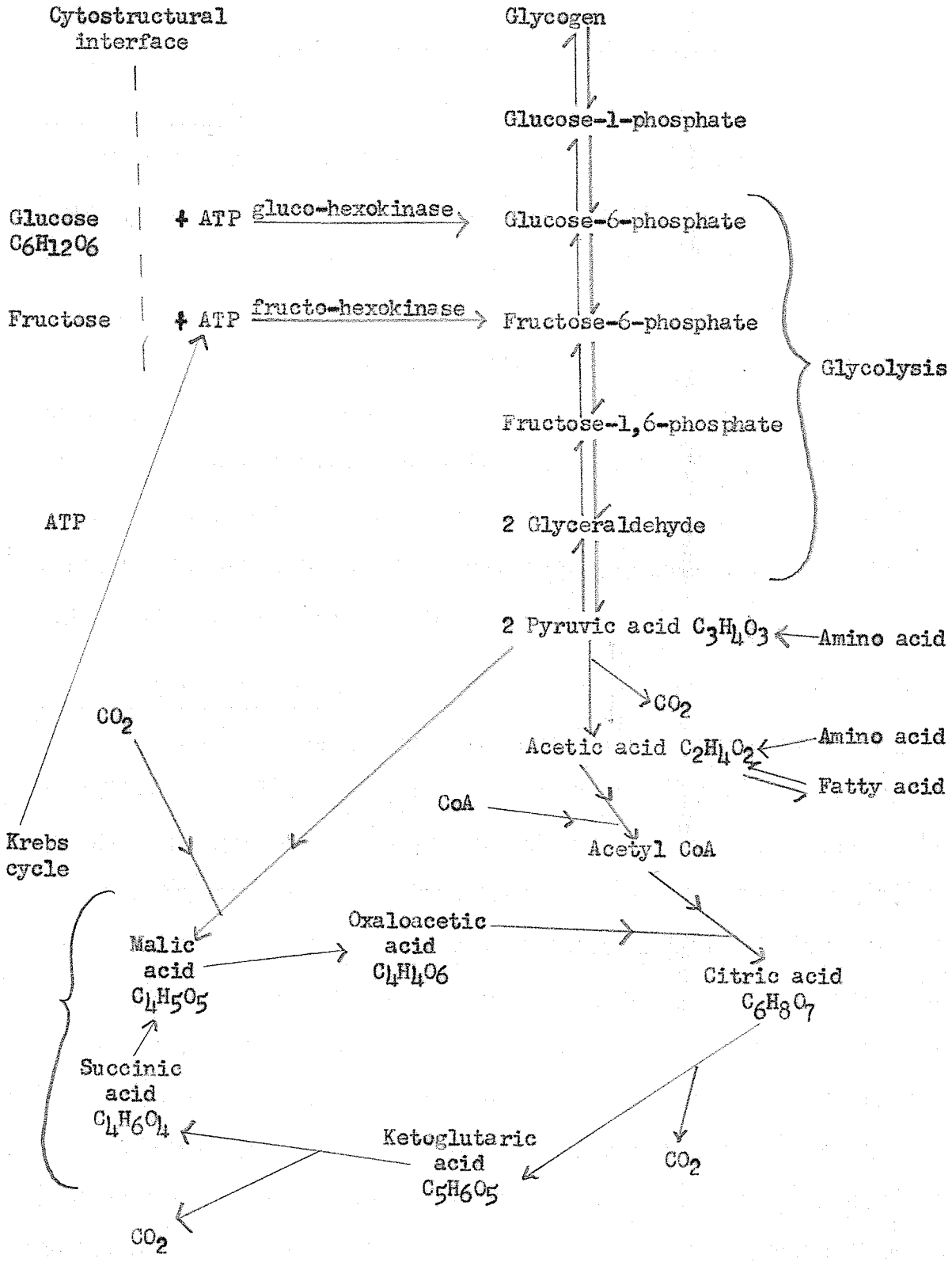

Figure 1. An Outline of Intermediary Metabolism.

glucose-6-phosphate is two mols of pyruvic acid. The glycolytic reactions are reversible and thus pyruvic acid can be converted to glucose-6-phosphate and glycogen. This end product of glycolysis, pyruvic acid, then undergoes oxidative decarboxylation to yield acetate which, after being activated by coenzyme A, condenses with oxaloacetic acid to enter the oxidative system known as the Krebs or citric acid cycle. In one complete revolution of this cycle there are three separate steps of oxidative decarboxylation which result in the oxidation of pyruvic acid to carbon dioxide and water, the regeneration of oxaloacetic acid and the liberation of energy which is stored in the high energy phosphate bonds of adenosine triphosphate or creatine phosphate to be used in the many energy-requiring reactions of the cell, including synthesis of protein and fat. The citric acid cycle is also the final common pathway for the oxidation of fats and proteins as these too can degrade to acetate. Alanine and some other amino acids can also be converted to pyruvic acid by the action of the enzyme amino acid oxidase. In this way, gluconeogenesis can occur, i.e. the synthesis of carbohydrate from non-carbohydrate precursors. The sugar fructose enters the glycolytic cycle by phosphorylation to form fructose-6-phosphate. This reaction is catalyzed by the enzyme fructo-hexokinase.

SITE OF ACTION:

From the metabolic relations which have been outlined, it can be seen that it is not inconceivable that a hormone such as insulin could exert a primary action upon carbohydrate metabolism which could have far-reaching secondary effects upon fat and protein metabolism.

In attempting to localize the site of the metabolic defect in diabetes, Chermick and Chaikoff (1) showed that the liver slices of alloxan diabetic rats oxidized glucose at a rate less than that of normal rats, while the oxidation of fructose in alloxan diabetic liver slices proceeded normally. Teng, Sinex, Deane and Hastings (2) found that, while the liver of alloxan diabetic rats was unable to synthesize glycogen from glucose at a normal rate, the synthesis of glycogen was unimpaired when pyruvate was used as a substrate. These findings suggested that the site of insulin action was somewhere before the pathways of fructose and glucose met, and did not involve the reactions leading from pyruvate to glycogen. Such a site is the formation of glucose-6-phosphate. Insulin could act at this point by speeding up the gluco-hexokinase reaction, but it could also act simply by promoting the entry of glucose into the cell so that it could be acted upon by the enzyme.

The Transfer Mechanism

Although it is not impossible that insulin has more than one site of action, the evidence to be summarized favors the concept that insulin acts by promoting the transfer of glucose into the cell and that such an action can explain all the other changes which have been observed to occur. Levine (3) has shown that in the eviscerated nephrectomized dog, insulin caused an increase in the volume of distribution of galactose. Since this preparation cannot metabolize galactose, it was concluded that the increased volume of distribution was brought about by the movement of galactose into the cells. It was inferred that insulin had a similar

action on glucose. Using the isolated rat diaphragm, Park (4) set out to demonstrate the accumulation of free glucose in the cell as a consequence of insulin action. In order to do this it was necessary to prevent the removal of this free glucose by hexokinase, and this he did by chilling the tissue, thereby slowing the enzyme activity. He was thus able to show that, whereas without insulin the amount of free glucose in the tissue corresponded to the amount in the extracellular space, when insulin was added the amount of free glucose was four to five times greater. He concluded that insulin had increased the rate of glucose transfer beyond the capacity of the chilled hexokinase system and therefore free glucose had accumulated within the cell. In the ciliary body of the rabbit's eye, insulin was clearly shown to enhance the permeability to glucose (5). By studying the rate of entry of glucose into the rat diaphragm at various temperatures, Levine and Goldstein (6) concluded that the changes in rate were such as to suggest that the transfer mechanism involved little or no energy exchange and was not under the influence of an enzyme. If so, this may be taken as evidence that any effect of insulin upon the transfer mechanism is a primary one and not secondary to changes in the availability of ATP.

According to this transfer mechanism concept of insulin action, the observed effects are explained as derivatives of the primary action in the following manner. The transfer of glucose into the cell is seen as resulting in more substrate being made available for phosphorylation by hexokinase, and thus for the synthesis of glycogen and the process of glycolysis. As glycolysis produces more pyruvate, more

substrate is made available for the citric acid cycle which then becomes capable of greater oxidation of acetate and production of high energy phosphate bonds. At the same time the increased rate of glycolysis permits more fatty acid synthesis from acetate, a process which seems to require the energy derived from glycolysis in order to proceed properly. As more acetate is utilized for oxidation and for fatty acid synthesis, less is converted into ketone bodies, and ketosis is thereby corrected. The ability of insulin to reduce gluconeogenesis is ascribed to the increased production of pyruvate from glycolysis, so that less is produced from the glycogenic amino acids such as alanine.

The Hexokinase Reaction

The second postulated site of insulin action is at the glucohexokinase reaction. This could be either a direct action upon the enzyme, or an indirect action resulting from an increased availability of ATP from the oxidative phosphorylations of the citric acid cycle.

Price, Cori and Colowick (7), using cell-free extracts containing muscle hexokinase, found that insulin was capable of releasing the inhibition imposed upon this system by anterior pituitary extracts and adrenal cortical extracts. However, Stadie and Haugaard (8) were unable to duplicate this finding and a direct stimulant effect of insulin upon the hexokinase reaction has not been shown conclusively. Indeed, apart from Price et al (7), no one has ever demonstrated that insulin is active in tissue in which cellular structure has been destroyed.

On the other hand, evidence has been presented which suggests that insulin acts by increasing the efficiency of oxidative phosphorylation, making available more high energy phosphate bonds to the hexokinase reaction and to fat and protein synthetic processes. Krebs and Eggleston (9) demonstrated that insulin accelerated the rate of oxidation of various members of the Krebs cycle by minced pigeon breast muscle. Actually insulin did not increase the initial rate of oxidation, but it slowed the decline in the rate so that after three to four hours the total amount of oxidation had been doubled. Stadie (10) points out that in tissue homogenates, such as were used by Krebs and Eggleston, glucose and glycogen break down to lactic acid and the hexokinase reaction ceases to operate. Thus these actions of insulin cannot be considered secondary to any change in the hexokinase reaction, but are interpreted as primary effects upon the citric acid cycle. These findings were confirmed by Shorr and Barker (11) and by Stare and Bauman (12) for pigeon muscle but not for mammalian muscle. Insulin effects on phosphorous metabolism have been shown many times. Kaplan and Greenberg (13) have demonstrated increased P^{32} turnover and increased ATP in rats treated with insulin. Haugaard, Marsh and Stadie (14) found a net increase in the number of mols of ATP produced in rat diaphragms incubated with insulin without any corresponding increase in oxygen consumption. They interpreted this as signifying an increase in the efficiency of oxidative phosphorylation but they have not eliminated the possibility that the extra phosphate bonds were derived from the process of glycolysis which

is anerobic. If this were the case, one would not expect an increase in oxygen consumption and the increased glycolysis could be ascribed to an increased formation of glucose-6-phosphate through either the transfer or the hexokinase mechanisms. However, in a recent review (15) Stadie mentions experiments which he had not previously reported, in which he and Vester demonstrated decreased ATP formation in the liver mitochondria of depancreatized cats. He does not state whether or not this decrease could be corrected by the addition of insulin in vitro but one would suspect not, considering the experiments of Baker, Chaikoff and Schusdek (16) in which the metabolic defects of diabetic liver slices could not be corrected by the addititition of insulin in vitro but only by the administration of insulin to the animal before killing. Therefore the concept of a primary insulin action upon the process of oxidative phosphorylation remains unproven.

SUMMARY:

Current evidence suggests that insulin acts by increasing the rate of transfer of glucose into the cell. The many effects of insulin, including lowering of blood sugar, stimulation of glycogen synthesis in liver and muscle, stimulation of glucose uptake by muscle, inhibition of ketone body production, stimulation of fat synthesis from acetate and the inhibition of gluconeogenesis can all be explained as secondary effects of this primary action.

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CHAPTER II. CURRENT METHODS OF INSULIN ASSAY

INTRODUCTION:

The discovery of insulin greatly advanced the treatment of diabetes mellitus and the knowledge of carbohydrate metabolism. Despite exhaustive study of the effects of the administration and the deprivation of this hormone, there is much that is yet to be determined about the amounts secreted, the concentration of the hormone in the circulating blood and in the peripheral tissues, and the factors controlling secretion. Clinically, it would be of importance to know whether spontaneous diabetes results from absolute insulin deficiency, from excessive action of insulin antagonists or from a combination of both factors. Similarly, in patients suffering from the effects of an abnormally low blood sugar, the precise metabolic defect is often not clear. The introduction of a method for the accurate estimation of small concentrations of insulin would be an important step toward the solution of many of these problems.

Insulin is a protein. There is no specific chemical test by which it can be measured. Biological assays have used the hypoglycemic effect in living animals and the stimulation of glycogen synthesis and glucose utilization in isolated muscle. A method of assay based upon an antigen-antibody reaction has recently been reported.

Biological Assay in Vivo

The assay used for commercial preparations of insulin is based on the production of hypoglycemic convulsions in intact mice. This method is not sufficiently sensitive to detect insulin in the concentrations in which it occurs in circulating blood.

To increase the sensitivity of the test, use was made of animals which had been deprived of their source of endogenous insulin and of known sources of hormones which have actions counter to those of insulin. In 1950, Bornstein (1) reported the use of the alloxan-diabetic, hypophysectomized, adrenalectomized rats. Although a measure of success was claimed, the reliability of the test was not demonstrated statistically. In addition, the animals were unstable and difficult to maintain.

Using alloxan-diabetic hypophysectomized rats, Beigelman and his co-workers in 1956 were unable to detect insulin in whole plasma (2), but were able to detect an insulin-like effect in certain plasma fractions (3).

Biological Assay in Vitro

In recent years, attention has been directed toward devising a suitable in vitro assay method. This method is based on the work of Gemmill (4), reported in 1940, which showed that insulin stimulated the utilization of glucose and synthesis of glycogen by the isolated diaphragm of the rat. Stadie and Zapp (5), in 1947, found that the effect was roughly proportional to the insulin concentration but with considerable scatter. In later work from the same laboratory, Marsh and Haugaard (6) limited the exposure of the diaphragm to insulin to one minute, a time which they found sufficient to produce measurable effects on the metabolism of the diaphragm for the following 90 minutes. From this they concluded that insulin became quickly bound to the diaphragm. If the diaphragm was exposed to a mixture of insulin and serum for one minute, it was found that the added serum reduced

the effect of the insulin present. They concluded that normal serum contains an insulin inhibitor.

Perlmutter and his group (7), in 1952, used the original method of having the insulin or serum to be tested in contact with the diaphragm throughout the test. In an effort to reduce the variability which they encountered, they adopted the practice of using, not the diaphragm of one rat, but pieces of the diaphragms from several rats in each flask. Nevertheless, their results were extremely inconsistent from day to day and from animal to animal. In attempting to circumvent this inconsistency, various modifications such as adrenalectomy of the rats, prior injection of adrenalin, use of other strains of white rats and use of the diaphragms of new-born rabbits were tried without success. Finally, without known changes in conditions or technique, the results became more consistent. Even with this improvement, they were still unable to detect insulin in normal human serum except shortly after the intra-venous injection of 20 units.

Groen and his co-workers in Holland (8), in 1952, also used the pooled diaphragm technique. Contrary to Stadie, they found that normal serum would stimulate the diaphragm to take up glucose, that serum from a mild diabetic had essentially the same properties and that serum from a patient in diabetic coma did not inhibit the stimulant effect of added insulin. Increased stimulant effect was reported from the serum of a patient with islet cell adenoma of the pancreas, while normal effect was reported from the serum of a patient with functional hypoglycemia with no tumor at operation. They measured the response to known concentrations of insulin and from the resulting data they constructed a concentration-action curve. However, it was found that the

slope of curves constructed at different times of the year differed from each other. Therefore, the estimates of plasma insulin concentrations were based on the comparisons with insulin standards run at the same time. From these comparisons, they concluded that the normal human serum insulin concentration is between 0.625 and 0.0625 milli-units (mU) per ml. However, this estimate is based on determinations on only two normal human subjects.

Randle (9), in 1954, reverted to the technique of using a single diaphragm in each flask. Although the responsiveness of the diaphragms varied markedly, he claimed that the slope of the dose-response curve was constant within certain limits and that by using a standard with each determination he could determine the plasma insulin concentration by reference to the dose-response curve. By this method, he estimated the mean normal post-prandial plasma insulin concentration to be 13 mU/ml. However, data presented later in the same paper did not produce a curve whose slope fell within the limits which he reported. This defect was acknowledged by Randle in a subsequent paper (10).

Vallance-Owen and Hurlock (11), using a method similar to that of Randle, reported in 1954 a dose-response relation, but did not give enough data for critical evaluation. They estimated the normal plasma insulin concentration to be 0.031 to 0.082 mU/ml.

It should be noted that none of these authors claimed to be able to measure anything more than net insulin activity in the plasma, i.e. the net effect resulting from the interplay of insulin and insulin antagonists.

Assay by Antigen-Antibody Reaction

Arquilla and Stavitsky (12) have recently utilized the antigenic properties of insulin as the basis for an assay. The amount of insulin present is estimated by the extent to which it inhibits the hemolysis of insulin-sensitized erythrocytes by a standard-titre antiserum. From the typical data given in the paper, the test appears capable of detecting 5.4 milli-units of insulin, with an error between duplicate determinations of 15%. However, detailed statistics are lacking, and the test has so far been applied only to pure solutions of insulin. Consequently, its usefulness as an assay of serum insulin concentration and its relation to insulin activity remain to be determined.

EXPERIMENTAL:

The biological assay of insulin was investigated using the method of Randle (9) and modifications thereof.

Methods

Male rats weighing 100-150 gm. were fasted 18-24 hours and then killed by decapitation. The diaphragm was quickly excised without bleeding and free of fat or fibrous tissue. One half of the diaphragm was placed in a buffer solution containing glucose in a concentration of 300 mg.% and the corresponding half from the same rat into another vessel containing the same solution with either insulin or serum added. The buffers used were either that described by Gey & Gey (13), a bicarbonate buffer (pH 7.4) which was equilibrated with 5% carbon dioxide and 95% oxygen, or that described by Tuerkischer and Wertheimer (14), a phosphate buffer (pH 7.4) which was equilibrated with 100% oxygen. Each hemidiaphragm was immersed in 4 c.c.

of incubation medium. The flasks were shaken in a water bath at 37°C. for measured lengths of time which varied from one to three hours. At the end of this time the hemidiaphragms were removed, blotted lightly and weighed. The media were analyzed for residual glucose content by the method of Somogyi (15). The difference between the original glucose content and the final glucose content of the medium constitute the glucose utilization of that diaphragm. The utilizations of the two hemidiaphragms from the same rat were compared to see how much of an effect, if any, had been produced by the insulin or serum.

Results

The results obtained are shown in Table I. It can be seen that, due to great variability and inconsistency, no satisfactory dose-response relationship could be demonstrated at the insulin concentrations used by Randle. At relatively high concentrations of insulin, of the order of one unit or more per ml., effects were readily demonstrable.

Various modifications of the method were tried in an effort to improve the results. Regarding the experimental animals, ad lib feeding or forced feeding rather than fasting prior to sacrifice did not affect results. Neither did placing the rats in a cold environment for 6-18 hours prior to killing, a measure which was suggested by Wertheimer's report (16) that it increased glucose utilization in the diaphragm. Results with imported rats were no better than those of our own inbred strain. Diaphragms from adrenalectomized rats were, if anything, less responsive. Killing the animals with curare or barbiturates rather than by decapitation brought no improvement.

TABLE I

DIFFERENCE BETWEEN GLUCOSE UTILIZATION OF RAT HEMIDIAPHRAGM INCUBATED WITH AND WITHOUT INSULIN

(Insulin effect expressed as mg. glucose / gram wet weight diaphragm / hr.) (Mean ± S.E.M.)

Insulin Concentration (milli-units per ml.)	Bicarbonate Buffer	Phosphate Buffer
1	-0.45 ± 3.22 (8)*	-0.21 ± 0.78 (6)
2	+5.57 ± 2.06 (10)	
3	+1.64 ± 0.49 (23)	
4	+7.18 ± 2.03 (6)	
5	+0.23 ± 0.62 (7)	+2.24 ± 1.63 (13)
10	-6.21 ± 1.39 (12)	+10.13 ± 3.76 (5)

* Number of determinations shown in parentheses.

Powdered amorphous insulin was no better than regular crystalline zinc insulin. The relative merits of using glucose concentrations of 300 or 600 mg.% were examined. As neither was superior from the standpoint of the insulin effect produced, it was decided to use the lower concentration as the amounts utilized would thus be more easily detected. The inclusion in the method of a period of equilibration in which the diaphragm is washed or soaked in buffer before being immersed in the incubation medium was not found to contribute anything to the accuracy of the method. No matter whether the preparations were incubated for one, two or three hours, the hourly rates of utilization showed approximately the same range of variation.

The results of a few trials using serum added to buffer are shown in Table II. It can be seen that duplicate determinations showed wide variation and that a stimulant effect could not be consistently demonstrated.

DISCUSSION:

Estimates of normal plasma insulin activity have ranged from that of Vallance-Owen and Hurlock (11), 0.031 - 0.082 mU/ml., to that of Randle (9), 13 mU/ml. Perlmutter (7) was unable to detect any insulin activity in normal human plasma. In addition to those discrepancies, all the methods have shown great variability with, in the case of Randle's experiments (9,10), unpredictable deviations from previously determined dose-response curves.

In the experiments reported here, using bicarbonate and phsophate buffers, neither insulin nor serum produced consistent stimulation of glucose utilization by the rat diaphragm.

TABLE II

DIFFERENCES BETWEEN GLUCOSE UTILIZATION OF RAT HEMIDIAPHRAGM INCUBATED WITH AND WITHOUT SERUM

Subject	Serum Dilution	Buffer	Serum Effect (mg. glucose/gm. wet weight diaphragm/hour)
A. Fasting	½	bicarbonate	≠0.7
			≠0.3
B. Post-absorptive	½	"	≠1.0
			≠1.6
			-3.5
C. Fasting	½	phosphate	≠3.72
			≠2.16
			≠3.95
			≠0.51
			-1.77
			≠3.21
B. Fasting	½	"	-14.04
			-10.94
B. Post-prandial (lipemic)	½	"	-9.75
			-6.45
			-6.80
			-2.55
			-36.40
			-10.25

Since, with this method, the best that can be hoped for is an indication of net insulin activity, it is possible for variations in the activity of insulin antagonists to mask the effects of any insulin present.

CONCLUSIONS:

In view of the great discrepancies between various authors' estimates of normal human plasma insulin activity, and because of the wide variability of results both in the literature and in the experiments reported here, it would appear that there has not been published as yet a method of plasma insulin assay which has been proven sufficiently sensitive and accurate for use in clinical research. It is postulated that variations in factors other than insulin which also influence glucose uptake are responsible for the inconsistency of the results obtained.

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CHAPTER III. THE MEASUREMENT OF ABSOLUTE SERUM INSULIN ACTIVITY

INTRODUCTION:

All the methods for the biological assay of insulin activity in serum or plasma which have thus far been reported (1-6) have reflected only net insulin activity, the result of the inter-action of insulin and insulin antagonists. The method to be reported herein has been devised in an effort to measure absolute serum insulin activity.

Du Vigneaud, Fitch, Pekarek and Lockwood (7) in 1931 and Wintersteiner (8) in 1933 reported that the amino acid cysteine, along with other sulfhydryl compounds, is capable of inactivating insulin by reducing the S-S linkages in the insulin molecule. Lens and Neutelings (9) reported that 0.25% cysteine reduced the activity of a 1% solution of insulin to 13-14% of its original value during one hour's incubation. Cysteine has also been showen to neutralize insulin in the presence of plasma (3-6).

METHOD:

The preparation employed was the isolated rat diaphragm. It was considered possible that the procedure of incubating the control diaphragm in a buffer solution and the test diaphragm in a dilute solution of serum in buffer so altered the osmotic relations of the diaphragm with its environment that its metabolic activity was impaired in an irregular fashion. Therefore the medium used consisted simply of the serum to be tested, with glucose added to bring the concentration to approximately 3 mg./ml.

This medium was divided into paired one-ml. portions. To one portion of each pair was added 4 mg. of cysteine HCl crystals. This

portion was then gassed with nitrogen to prevent oxidation of cysteine to cystine. Both media were then incubated in a water bath at 37°C. for one hour. At the end of the hour, an ad lib fed male rat weighing 100-150 grams was killed by decapitation. The two leaves of the diaphragm were quickly dissected out, weighed and immersed in the incubation media. The flasks were then gassed with 5% carbon dioxide and 95% oxygen and incubated in a water bath at 37°C. for one hour. At the end of the hour the glucose contents of the flasks and of the original incubation medium were determined by the method of Somogyi (10). The difference between the glucose utilization of the hemidiaphragm in unmodified serum and that of the corresponding hemidiaphragm in serum pre-treated with cysteine was attributed to the insulin content of the serum. This was expressed as mg. glucose utilized per gram wet weight diaphragm per hour. No attempt was made to express this as units of insulin.

Tests were made upon serum from clinically normal subjects in the fasting state, following a glucose meal and following injection of insulin. An infant with idiopathic hypoglycemia and an adult with diabetic acidosis were also studied.

The effect of adding insulin in vitro was determined. Crystalline zinc insulin powder (Connaught) without preservative was dissolved in 0.01 N H_2SO_4 and added to aliquots of serum to produce the desired concentration. To 5 ml. of serum 0.1 ml. of the insulin solution of appropriate concentration was added.

RESULTS:

Normal Fasting Serum Insulin Activity

Table III shows the fasting serum insulin activities of twelve clinically normal adult subjects. The figures shown represent duplicate

TABLE III

FASTING SERUM INSULIN ACTIVITY OF CLINICALLY NORMAL SUBJECTS

Subject	Sex & Age	Glucose Utilization (mg./gm.wet wt./hr.) Serum	Serum + Cysteine	Difference	Mean Difference (Insulin Activity)	Variation of Duplicates from Their Means
I.S.	M 27	5.20	0.00	5.20		
		2.71	0.00	2.71	3.96	1.25
M.D.	M 40	6.38	3.07	3.31		
		8.30	4.15	4.15	3.63	0.32
S.D.	M 30	8.33	6.32	2.01		
		14.05	9.54	4.51	3.26	1.25
M.S.	F 58	5.50	2.49	3.01		
		4.86	1.50	3.36	3.18	0.18
B.E.	F 25	4.54	1.13	3.41		
		5.18	2.27	2.91	3.16	0.25
D.I.	F 23	7.74	5.11	2.63		
		8.57	5.64	2.93	2.78	0.15
B.C.	M 23	7.08	4.10	2.98		
		7.75	5.66	2.09	2.54	0.45
W.E.	M 29	3.24	0.00	3.24		
		1.66	0.00	1.66	2.45	0.79
H.F.	M 22	3.38	2.24	1.14		
		4.15	1.60	2.55	1.85	0.71
G.W.	M 26	2.06	1.10	0.96		
		2.19	0.00	2.19	1.58	0.61
C.P.	M 12	1.98	0.00	1.98		
		0.55	0.00	0.55	1.27	0.71
W.P.	M 37	3.26	3.42	0.00		
		1.75	0.00	1.75	0.88	0.88
					Mean 2.54	Mean 0.63
					S.D. ±1.00	S.D. ±0.38

determinations on the same specimen of serum. The mean of this sample was 2.54 ± 1.00 (standard deviation) mg./gm./hr. In two subjects, duplicate determinations differed from their means by over 2.00 mg./gm./hr. These subjects were omitted from the table. In two subjects, W.E. and G.W., repeat fasting determinations were done on different days. As seen in Table V, the values obtained were essentially unchanged.

The Effect of In Vitro Addition of Insulin

Table IV shows the effect of the in vitro addition to the serum of zinc insulin crystals in concentrations of 75, 150, and 300 mU/ml. With 75 mU/ml., the mean increase observed did not differ significantly from zero. With 150 and 300 mU/ml., progressive increase in serum insulin activity were noted. The increased produced by 150 mU/ml. is significant at the 2% level; the increase produced by 300 mU/ml. is significant at the 1% level. In Figure 2 are plotted the mean increments in glucose utilization together with their standard errors for each added amount of insulin. The points thus plotted can be joined by a straight line.

The Effect of Ingestion of Glucose

Two subjects were assayed in the fasting state and after the ingestion of 50 gm. glucose (Table V). In G.W. the serum insulin activity increased from 1.09 to 3.77 mg./gm./hr. In W.E. the serum insulin activity increased from 2.25 to 8.98 mg./gm./hr.

The Effect of Intra-Venous Injection of Insulin

In subject D.I., the fasting serum insulin activity was found to be 2.78 mg./gm./hr. Immediately after the fasting specimen was drawn, 15 units of regular insulin was injected intra-venously. A second

TABLE IV

THE EFFECT OF THE IN VITRO ADDITION OF INSULIN ON THE SERUM INSULIN ACTIVITY

Increase in Glucose Utilization (mg./gm./hr.)

Amount of Insulin Added: (milli-units/ml.)	75	150	300
Subject			
A.N.	2.04	2.34	3.01
H.F.		0.33	2.88
S.C.	0.77	2.07	5.33
G.W.	0.88	2.45	2.47
I.S.	0.16	0.92	2.09
MEAN $\pm$ S.E.M.	0.96 $\pm$ 0.42	1.62 $\pm$ 0.42	3.16 $\pm$ 0.56

TABLE V

THE EFFECT OF THE INGESTION OF GLUCOSE ON
THE SERUM INSULIN ACTIVITY

Subject	Specimen	Serum	Serum + Cysteine	Difference	Mean Difference (Insulin Activity)	Effect of Glucose Ingestion
G.W.	Fasting	3.49	2.90	0.59		
		6.45	4.85	1.60	1.09	
	1 hour after	4.80	0.00	4.80		
	50 g. glucose	2.74	0.00	2.74	3.77	2.68
W.E.	Fasting	4.84	2.59	2.25		
	1 hour after	17.95	9.89	8.06		
	50 g. glucose	13.00	3.10	9.90	8.98	6.73

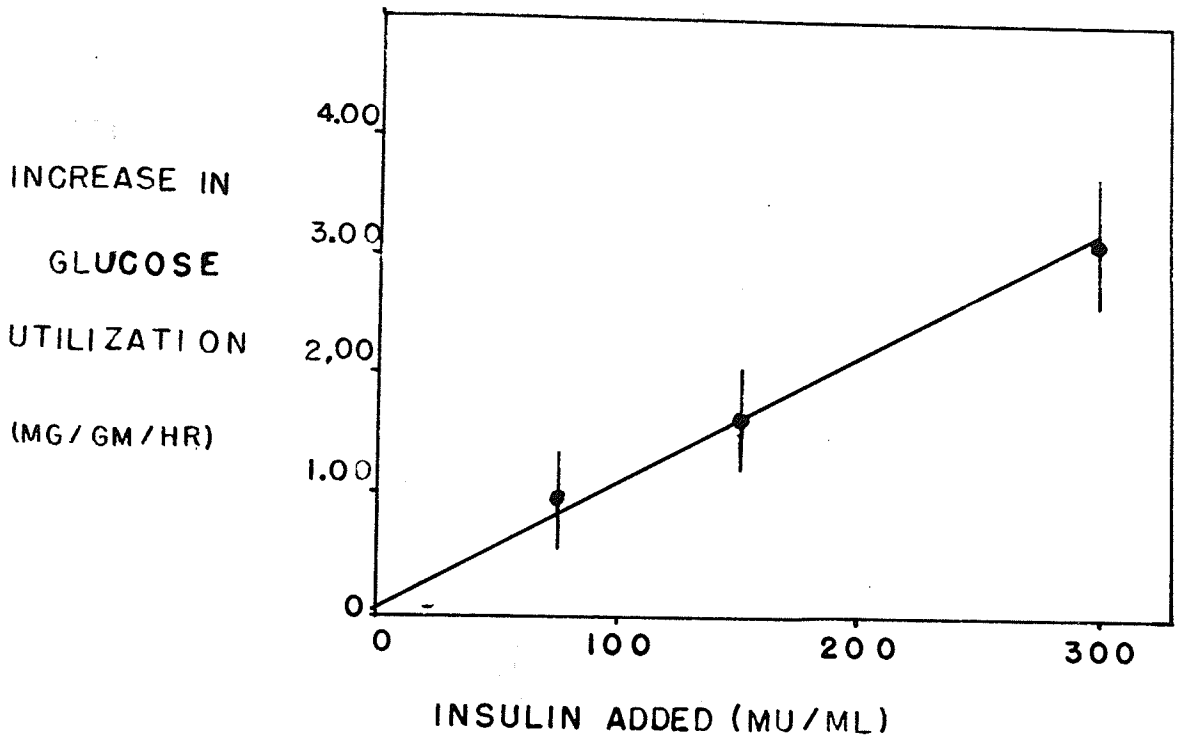

Figure 1. The Effect of the In Vitro Addition of Insulin on the Serum Insulin Activity.

(⊢●⊣ Mean ± S.E.M.)

serum sample drawn five minutes later showed an insulin activity of 1.32 mg./gm./hr.

In subject W.P., the same procedure was used except that the second serum sample was drawn from the opposite arm 45 seconds after the insulin injection. The amount of insulin used in this subject was 20 units. In this case, the serum insulin activity rose from a pre-injection level of 0.61 mg./gm./hr. to a post-injection level of 3.16 mg./gm./hr.

Idiopathic Spontaneous Hypoglycemia of Infancy

The serum insulin activity of a 3½-month old female with idiopathic hypoglycemia was 4.42 mg./gm./hr. This value was higher than the fasting value obtained on any other subject. Glucose concentration of this same specimen of serum was 40 mg.%. Treatment with ACTH successfully prevented convulsions but did not bring the blood sugar into the normal range. One hour after ACTH injection, the serum insulin activity was 4.81 mg./gm./hr.

Serum Insulin of a Diabetic After Withdrawal of Insulin

A 67-year old male who had been diabetic for nineteen years showed a serum insulin activity of 1.74 mg./gm./hr. 44 hours after receiving his last injection of regular insulin. After another 24 hours had elapsed, no serum insulin activity was demonstrable. At this time the patient was ketotic.

DISCUSSION:

The ability of cysteine to inactivate insulin has long been known, but this phenomenon has never before been used to aid in the estimation

of the amount of insulin activity present. The only difference between the medium without cysteine and that with cysteine is that the latter has had any contained insulin inactivated. Since Randle (5) reported that cysteine had no influence upon the uptake of diaphragms incubated without insulin, the differences observed between the corresponding hemidiaphragms may be attributed to the insulin in the serum. Although there remains the variation of the individual rat's susceptibility to insulin, the masking effect of other factors is eliminated and there results an estimation of absolute rather than net insulin activity.

Evidence supporting the contention that this is indeed a measure of insulin activity are the findings of increased insulin effects within one minute after the intra-venous injection of insulin, after the addition of insulin to serum in vitro and after a carbohydrate meal. Further evidence is found in the disappearance of insulin activity from the serum of a diabetic 68 hours after the last injection of regular insulin.

That such factors as ACTH which alter net insulin activity do not affect absolute insulin activity as measured by this test is suggested by the finding of similar insulin activity before and after the injection of ACTH.

The in vitro addition of increasing amounts of insulin produces progressive increases in the serum insulin activity. The means of the increments, when plotted against the amount of insulin added, fall on a straight line. This is in contrast to the report of Randle (5) who found the cube root of the uptake to be proportional to the logarithm of the insulin concentration.

The precision of the method is indicated by the variations of duplicate determinations from their means as listed in Table III.

These variations have a mean of 0.63 with a standard deviation of 0.38 mg./gm./hr. The size of the variations showed no relation to the size of the observed value. Whether the range of variation encountered is permissible will depend upon the size of the differences observed under various experimental conditions.

The estimates for normal human plasma insulin activity thus far reported range from a low of 0.031 mU/ml. reported by Vallance-Owen and Hurlock (6) to a high of 13 mU/ml. reported by Randle (5). Since these values represent net insulin activity, they are not comparable to the values for absolute insulin activity which are given by this method. From the graph in Figure 2, the mean normal human insulin activity of 2.54 mg./gm./hr. would represent an insulin concentration of 225 mU/ml. if the straight-line relationship holds true throughout the range of insulin concentrations.

The findings of a serum insulin activity above the range of normal in the infant with spontaneous hypoglycemia suggests that this subject may have an excess of insulin in the serum. However, it will be necessary to study normal infants before this value can be considered definitely abnormal.

SUMMARY AND CONCLUSIONS:

A method has been proposed for absolute serum insulin assay whereby use is made of the property of cysteine to inactivate insulin. Diaphragms are incubated in pure serum with and without cysteine. The differences in glucose utilization are taken as an index of absolute insulin activity.

Preliminary results suggest that this is a valid measure, being increased by the addition of insulin in vivo and in vitro, and by the

ingestion of a glucose meal, and being decreased following the withdrawal of insulin from a diabetic of long-standing.

The mean value for the absolute serum insulin activity of normal fasting adults is 2.54 mg./gm./hr. which corresponds to a serum insulin concentration of 225 mU/ml.

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