THE RAT DIAPHRAGM METHOD AND PLASMA INSULIN ESTIMATION

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In spite of the great success achieved in the treatment of diabetes mellitus the pathogenesis of the condition remains obscure. Since the discovery of insulin by Banting and Best in 1921 it was assumed that the condition was due to insulin deficiency. Recent evidence suggests that diabetes mellitus is a syndrome rather than a disease entity which can result also from other hormonal disturbances. Estimation of insulin in the plasma of normal and diabetic persons would therefore help clear the doubt. Hence many workers notably, Bornstein, Vallance-Owen and Hurlock and Randle have tried to evolve a method for the assay of insulin in the plasma.

In the absence of a quantitative chemical or physical method insulin has to be assayed biologically. The present standard methods for the assay of insulin require relatively high quantities of insulin, viz, 1 unit. The plasma contains much smaller amount of insulin per ml. Therefore a more sensitive method of assay is needed.

Hemmingsen, Nielsen and Nielsen (1938) demonstrated that adrenalectomised mice developed hypoglycemic convulsions with 0.0001 to 0.0002 unit of insulin per 10 Gm. of body weight. Gellhorn *et al* (1941) found the convulsive dose of insulin in adrenodemedullated hypophysectomised rats to be 0.001 unit per 100 Gm. of body weight. Anderson, Lindner and Sutton (1947) demonstrated that in adrenodemedullated alloxan diabetic, hypophysectomised (ADH) rats 0.00012 unit of insulin given intravenously caused a significant fall in the blood sugar (see also Anderson and Long 1947a and 1947b). They also showed that the dose response curve had a linear relationship between the arithmetic value of the change in the blood sugar and the log. of the dose of insulin. Bornstein (1950) and Bornstein and Trewhella (1950) used alloxan diabetic, hypophysectomised, adrenalectomised (ADHA) rats for the assay of plasma insulin and reported good results. Beigelman *et al* (1956) have studied hypophysectomised alloxan diabetic rats (HAD rats) for assaying insulin.

The increased glucose uptake of the isolated rat diaphragm was used for the assay of plasma insulin by various workers (Groen *et al*, 1952; Vallance-

Owen and Hurlock, 1954; Vallance-Owen, Hurlock and Please 1954 and 1955; Randle 1954a, 1954b, 1954c. Aiman and Kulkarni 1958). Balmain *et al* (1954) demonstrated that the gas output of the lactating mammary gland was related to the amount of insulin. Bleehan and Fisher (1954) showed that insulin increases the glucose utilisation of the isolated mammalian heart. These latter two methods have not so far been used for plasma insulin estimation. Recently Robinson and Kitty (1952) and Porter (1953) have tried to separate insulin from protamine and other proteins by paper chromatography but it is too early to comment on this method.

Of these various methods the ADHA rat and the rat diaphragm have been extensively studied. Randle (1957) observed that the ADHA rat, apart from being difficult to prepare was a delicate and unstable preparation. Randle (1954c) also demonstrated that this preparation was much less sensitive to insulin than the rat diaphragm.

Gemmill (1940, 1941) was the first to show that glycogen synthesis of the rat diaphragm increased with the addition of insulin to the incubation medium. He also worked with the frog's sartorius muscle and found that insulin did not increase the glycogen synthesis of that muscle. Since then many workers have tried to utilise this observation for the purpose of estimating plasma insulin (see references above). A great deal of work has yet to be done to study the metabolism of the rat diaphragm with particular reference to the factors which affect the glucose uptake of the rat diaphragm and the effect of insulin on it. It is the purpose of this review to collect together the information regarding this method so that a suitable modification of this method may be evolved which can reliably estimate the plasma insulin concentration—an ideal which is yet to be achieved.

THE RAT DIAPHRAGM METHOD

In its simplest form the method is as follows—A white rat of a particular sex is starved over a period of 20 to 24 hours and killed by stunning or decapitation. A longitudinal incision is made on the abdomen extending from the xiphisternum to the pubis. The liver is pulled down and the ligaments attaching it to the diaphragm are cut. A small puncture is made in the diaphragm which then balloons out. Peripheral attachments are then severed by small nicks with the scissors and the whole of the diaphragm except the tendinous posterior portion is removed with as little trauma to the tissue as possible, care being taken to see that the great vessels in the abdomen are not injured. After removal it is gently pressed between filter papers. The serrated edges are trimmed and it is then divided into 2, 4 or 6 pieces. One or more of such pieces are placed in the flask containing the incubation medium with glucose concentration varying between 0.1 percent to 0.5 percent. The flasks are either Warburg's vessels when that apparatus

is used or small beakers when any other mechanical shaking incubator of the Dubnoff type is used. The gas phase in the flasks is either oxygen, mixture of oxygen and carbon dioxide or ordinary air. The temperature of incubation is set at 36°C to 38°C. After a fixed incubation period the flasks are removed, the diaphragms are blotted dry and weighed and glucose in the medium estimated by one of the standard methods. The difference between the pre-incubation and the post-incubation glucose content of the medium is taken as the glucose uptake of the rat diaphragm. This glucose uptake is further calculated as mg. of glucose per Gm. wet weight of the diaphragm per hour ; or mg. of glucose per 10 mg. of dry weight of the diaphragm per hour ; or mg. of glucose per unit weight of the diaphragm per 100 ml. of the medium per hour. The increase produced in this value by insulin is designated as insulin effect and that produced by plasma is termed the plasma effect.

Originally Gemmill (1940) determined the initial and final glycogen content of the diaphragm and designated the insulin effect as the increase produced in glycogen synthesis. Villee and Hastings (1949) using labelled glucose showed that only a part of the glucose taken up by the diaphragm is converted to glycogen, the rest of it is converted to lactic acid or to some unknown products. They also showed that only a part of the synthesised glycogen is derived from the glucose in the medium. Bartlett, Wick and Mackay (1949) also found that a small fraction of the labelled glucose utilised by the diaphragm appeared as glycogen. Perlmutter *et al* (1952) trying to estimate serum insulin noted that the glucose utilisation of the diaphragm had no relation to its glycogen synthesis. The glucose uptake had more definite quantitative relation to the concentration of insulin in the medium. In the diaphragm glycogen is constantly being formed from its precursors and is broken down to various intermediary products except glucose. As the muscle does not contain the enzyme glucose-6-Phosphatase (Cori *et al*, 1938; Villee *et al*, 1952) it can not release free glucose back into the medium. Therefore, the uptake of glucose by the diaphragm should be the more reliable in estimating insulin effect.

Various factors, a detailed description of which follows, affect the glucose uptake of the rat diaphragm and the insulin effect. As mentioned above it has not been possible to estimate the plasma insulin concentration very accurately by this method as the precision is not high and the specificity of insulin effect in the plasma is questioned. It is therefore necessary to review the work done so far and evolve a suitable modification of the method which will give it the necessary precision and specificity for plasma insulin estimation.

THE FACTORS INFLUENCING THE GLUCOSE UPTAKE OF THE ISOLATED RAT DIAPHRAGM AND THE 'INSULIN EFFECT'

The Rat: Healthy albino rats belonging to a homogenous weight group should be selected. The particular strain of rats should be strictly adhered to in a given series of experiments as it has been widely reported that different strains of rats vary in their sensitivity to insulin. Using the Haffkins strain we (Aiman and Kulkarni, 1957 and 1958) could fairly easily obtain the insulin effect. Gray (1957) could not obtain any insulin effect on the diaphragms of his rats. Randle (1957) and Vallance-Owen (1957) maintain that if a responsive strain is selected for this work the insulin effect is obtained without much difficulty.

Diaphragms of rats weighing below 80 Gm. give inconsistant results (Stadie and Zapp, 1947; Krahl and Park, 1948) and those of rats weighing above 200 Gm. are probably too thick to permit complete diffusion.

Starving of the animals 20 to 24 hours prior to killing is essential. With fed animals the basal glucose uptake is high and when the basal glucose uptake is high the insulin effect is low (Krahl and Park, 1948). If glucose is given orally to the animals an hour before killing them the glucose uptake of their diaphragms is higher presumably due to increased insulin content of such diaphragms. Insulin content of the diaphragms from the normal rats should be variable and for this reason it would be interesting to see whether use of diabetic rat diaphragms gives more consistent results.

Previous feeding of the rats affects the glucose uptake of their diaphragms. Glucose uptake is less when the rats are force fed fat diet for four weeks (Gilmore and Samuels, 1949). Previous feeding of high corbohydrate diet increases the glucose uptake of the rat diaphragm; previous fat feeding reduces it but the insulin effect is unaffected (Hansen *et al*, 1951).

The environmental temperature in which the rats are housed prior to their sacrifice affects the glucose uptake of their diaphragms. Wertheimer *et al* in 1954 observed that the diaphragms of rats exposed to a cooler environment utilised more glucose than those of rats exposed to warmer temperature. They noted that it was the change from the room temperature rather than the absolute temperature which was responsible for the change in the glucose uptake. Baker and Sellers (1953) obtained results to suggest that cold acclimatisation of rats increased their metabolic rate and the tissue utilisation of glucose. This factor has to be borne in mind when the animals are housed in airconditioned room and the experiments are performed at room temperature.

The Diaphragms: Perlmutter *et al* (1952), Groen *et al* (1952) and others have shown that on the weight basis smaller diaphragms utilise more glucose

per Gm. of their weight than the larger diaphragms. Hence the glucose uptake of quarter diaphragms is more than that of the hemidiaphragms. Abrahams *et al* (1953) found that cutting the hemidiaphragms into 3 or more pieces was unreliable which is contrary to the finding of Leupin and Verzer (1950) who obtained uniform results when the hemidiaphragms were divided into 3 pieces. We have obtained fairly consistant results with 1/6 of a diaphragm when 3 such pieces were pooled in one flask (unpublished data). This is in conformity with the findings of Perlmutter and Greep (1948). Pooling of the diaphragms in a flask increases the total weight of the tissue per flask. This minimises the error in glucose estimation when the uptake is calculated per Gm. of the tissue. When hemidiaphragms are used such a pooling is imperative in order to minimise the biological variation of the diaphragms removed from different rats. Liebecq (1954) has rightly emphasised the point that the weights of the tissue per flask should be as nearly equal to their mean weight as possible; because the smaller the weight of the tissue in the flask the greater is the glucose uptake per Gm. of the weight. Unequal distribution of the tissue in different flasks might vitiate the results in estimating plasma insulin levels.

According to Stadie and Zapp (1947) initial glycogen content of the diaphragm does not significantly affect the insulin effect on the diaphragm. Hanson *et al*, (1951) have shown that if the initial glycogen content of the diaphragm was higher than normal, the glucose uptake was lower. Perlmutter and Greep (1948) noted that prolonged fasting–which might lead to depletion of muscle glycogen—increased the glucose uptake of the rat diaphragm. The diaphragms of diabetic rats utilise less glucose but the insulin effect is unaffected (Krahl and Cori, 1947).

Brown *et al* (1952) have studied the effect of soaking the diaphragm in the medium at 37°C, at room temperature, and at a temperature lower than the room temperature prior to incubating them. Their results show that (a) previous soaking increases the glucose uptake of the rat diaphragm, (b) this increase is maximum when the temperature of the soaking medium is 37°C and, (c) this increase reaches its maximum after 10 minutes of soaking.

When the diaphragms are incubated in a medium which contains glucose in a concentration higher than the physiological one (as is usually the case) some glucose enters into the extracellular space passively and hence false higher values for the glucose utilisation may be recorded. This can be prevented by soaking the diaphragms in a medium containing glucose in the same concentration as in the incubation medium for a period of ten minutes and then blotting them dry and suspending in the incubation medium.

The diapharagms should be removed with due care and the serrations on the edges should be trimmed. The serrations on the edges increase the cut

surface of the diaphragm which increases the glucose uptake. Injury to the great vessels in the abdomen of the rat before removal of the diaphragm causes the diaphragm to be bathed in blood which should be avoided. It is a good practice to weigh the diaphragms at the end of the incubation period as it prevents much unnecessary handling of the tissue prior to incubation. One disadvantage of such a procedure is that one cannot be sure of uniform distribution of the tissue in different flasks; but with experience this difficulty can be overcome.

The Medium: Any medium which has a composition very close to that of the extarcellular fluid should be suitable. Gey and Gey (1936) used one such medium for the study of the growth of tumor cells. This medium is chiefly used at present for the study of plasma insulin effect. Gemmill (1940, 1941) used a slight modification of this medium. Apparently Stadie and Zapp (1947) simple phosphate buffer gives fairly good results (Aiman and Kulkarni, (1958). Tuerkischer and Wertheimer (1948) also used simple phosphate buffer and observed that the glucose uptake was not hampered in any way. Though, practically any medium having isomolarity of the plasma can be used it is abvisable to use Gey and Gey's medium in order to achieve uniformity of method and also to enable us to compare the data reported by various workers. We emphasise this point because various electrolytes in the medium affect the glucose uptake of the rat diaphragm and the effect of insulin on it. Stadie and Zapp (1947) reported interesting findings in this respect. They observed that maximum insulin effect was obtained in a medium which contained no potassium. With increase in the potassium concentration there was a linear decrease in the insulin effect, till it reached zero when potassium concentration in the medium was 75 percent of the total electrolytes. When potassium was the only cation in the medium the glucose uptake was quite low. If Phosphorus was absent or was present in a concentration of 0.05 M in the medium there was no change in the glycogen synthesis but higher concentrations depressed it. Optimum magnesium concentration was 0.005 M to 0.01 M. Higher or lower concentrations depressed the glycogen synthesis of rat diaphragm. With regards to potassium similar results were obtained by Tuerkischer and Wertheimer (1948). Potassium exchange in the isolated rat diaphragm was studied by Kamminga *et al* (1950), and Calkins *et al* (1954). Tuerkischer and Wertheimer (1948) further noted that the calcium and the ammonium ions had inhibitory effect on the glucose uptake of the rat diahragm and the insulin effect.

The pH of the medium should be adjusted to 7.4. Stadie and Zapp (1947) noted that between pH 6.3 and pH 7.6 there was no change in the glycogen synthesis, but the final pH tended to come towards 6.8. They therefore used the medium with pH 6·8. Tuerkischer and Wertheimer (1948) on the contrary found no change in the final pH of the medium when

it was initially at pH 7.4. They found that at pH 6.8. glycogen synthesis was depressed by 13 ± 7 per cent. Mackler and Guest (1953a) observed that the rate of phosphorylation of glucose was decreased when pH of the medium was changed from 7.5 to 7.0.

The glucose concentration in the medium influences the uptake of the diaphragm; when it is less than 0.1 percent the uptake per Gm. is erratic (Stadie and Zapp, 1947). The glycogen synthesis then steadily increases as the concentration of glucose in the medium increases from 0.1 per cent to 1.0 percent. Gemmill and Hamman (1941) showed that insulin effect was more at glucose concentration of 0.3 to 0.5 percent than at 0.2 percent. We feel that the concentration of glucose in the medium should be near 0.25 percent which could be easily obtained in the plasma medium mixtures (as discussed below) when the blood sugar ranges from 0.1 percent in the normal persons to 0.5 percent in severely diabetic patients.

For the plasma insulin estimation plasma can be used as such or the plasma medium mixtures may be used. The plasma medium mixtures have two advantages over the undiluted plasma. Firstly, by preparing such a mixture the change in the electrolyte composition of plasma from that of the medium can be diminished, and hence the insulin effect obtained by adding a known concentration of insulin to the medium can be compared with the effect of insulin in the plasma—the 'plasma effect'. Secondly, the glucose concentration in the medium and that in the plasma medium mixtures can be kept constant throughout the series of experiments. If undiluted plasma is used this can be done only at a very high concentration of glucose in the medium. One disadvantage of preparing the plasma medium mixtures is that we do not know as yet how far this dilution of the plasma affects the activity of insulin and its antagonists. There is some evidence to suggest that insulin activity changes with the dilution of the plasma (Takeuchi *et al,* 1957; Wright, 1957). Much more work will have to be done in order to determine the effects of dilution and to obtain the critical dilution of the plasma which will give uniform and possibly absolute plasma insulin levels (see below).

The quantity of the incubation fluid does not seem to make any difference to the glucose uptake. However, the smaller the quantity of the incubation fluid the greater will be the difference between the initial and the final glucose concentration and hence the less will be the error in determining the actual uptake of glucose by the diaphragm. Usually 1 ml. to 4 ml. of the medium are used.

The Incubation: The incubation can be carried out in a suitable apparatus which permits of maintaining uniform temperature and a suitable gas phase with constant shaking. Warburg's apparatus or the Dubnoff metabolic shaking incubator is usually used. The temperature of incubation is set at

36° to 38° C. Most workers prefer 95 percent oxygen and 5 percent carbon-dioxide mixture for the gas phase. Krahl and Cori (1947) noted that when the gas phase was 95 percent nitrogen the insulin effect was depressed by 90 per cent. Walaas and Walaas (1952) obtained insulin effect on the isolated rat diaphragm when incubated in strictly anaerobic condition though this was less than when the gas phase was oxygen. Demis and Rothstein (1954) found no insulin effect when the incubation was carried out in an anaerobic condition. They added insulin to the medium after making the tissue anaerobic for 15 minutes. Walaas and Walaas (1952) had added insulin immediately after making the tissue anaerobic. Ottaway (1955) observed that insulin increased the glucose uptake of the rat diaphragm in first 15 minutes when incubated anaerobically but thereafter it was ineffective. All the workers agree that the basal glucose uptake is the same under aerobic and anaerobic conditions. We used 100 percent oxygen as gas phase in our previous work (Aiman and Kulkarni, 1958). At present we are using ordinary air as gas phase and getting quite a good insulin effect for the different concentrations of insulin.

Increase in the rate of shaking increases the glucose uptake of the rat diaphragm (Brown *et al*, 1952.) It is, therefore, absolutely essential that the rate of shaking be kept constant in a series of experiments. This is especially important in case a standard insulin curve is constructed to determine the value of the insulin effect and the plasma effect.

The duration of the incubation affects the glucose uptake when the latter is expressed as mg. of glucose per Gm. of the weight of the diaphragm per hour. Park *et al* (1952) have shown that it is maximum when the incubation period is 30 minutes. It then gradually falls during the subsequent 30 minute periods. Many workers (Haft *et al*, 1953) have observed that the glucose uptake is greater during the early periods of incubation. For plasma insulin estimation the incubation is carried out for a period of 2 to 4 hours.

Seasonal variation: Groen *et al* (1952) have reported that during the summer of 1950 the sensitivity of diaphragms to insulin was particularly low. Since then many workers have observed that during the hot summer days the results tend to be erratic. Wertheimer *et al* (1954) noted that there was no seasonal variation in the glucose uptake of the rat diaphragm except in very hot summer days when the insulin effect might be low. We have worked in March and early April (Aiman and Kulkarni, 1957) and have obtained good insulin effect for one concentration of insulin viz, 10^{-3} unit per ml. These days must surely be warmer than the hot summer days in the temperate zone. As stated previously much might depend on the particular strain of rats used. Though there is considerable evidence to suggest that the daily variation in the glucose uptake of the rat diaphragm may be high, the insulin

effect remains fairly constant. As long as satisfactory insulin effect is obtained work may be carried out in any season.

The final estimation of glucose in the medium must be very accurate; because a slight error in this will be highly magnified in the final results and might vitiate the whole data. As discussed above there are many factors which affect the glucose uptake of the rat diaphragm. Therefore it is very important that one follows a rigid technic and pays due attention to the minutest details.

Adaptation of the rat diaphragm method for plasma insulin estimation: At present most of the workers use the pooled hemidiaphragm technic. In this, several vessels, each containing 2 to 8 hemidiaphragms are incubated. One of the vessels containing the medium alone acts as a control. At least three of the vessels contain insulin in the medium in three different concentrations. One or more vessels contain plasma, plasma medium mixture, or the extract of plasma. At the end of the incubation period the glucose uptake in each vessel is determined on weight basis per hour. A graph is then plotted with increase in the glucose uptake produced by insulin against the log of the respective insulin concentration. The increase produced by the plasma is marked on this graph and the insulin concentration calculated therefrom. Randle maintains that a better straight line can be drawn by plotting the cube root of the insulin effect against the log of the respective insulin concentration. Vallance-Owen and Hurlock obtained a perfect straight line response when the insulin effect was plotted against the cube root of the respective insulin concentration. Any transformation is permissible which will give a perfect straight line.

Stadie (1951) and Marsh and Haugaard (1952) have studied the insulin binding of the rat diaphragm. They observed that exposure of the diaphragm to the insulin containing medium for a short period increased its glucose uptake. Exposing the diaphragm to the plasma for a short time before incubating in the plain medium would give the plasma effect. But the difference between the plasma glucose concentration and that in the medium would interfere with the accuracy of the final results. When the diaphragm is exposed to higher glucose concentration some glucose enters into the extracellular space passively and at least 10 minutes vigorous shaking is necessary to remove that glucose (Bornstein and Park, 1953). It is therefore a good practice to adjust the glucose concentration of the medium and the plasma medium mixture beforehand and to incubate the diaphragms in the plasma medium mixtures and insulin medium mixtures.

Many times a referenee graph is plotted from the means of several observations and in subsequent studies only one concentration of insulin is used to ascertain the insulin effect on that day and the plasma effect is

marked on this readjusted graph. Sometimes two insulin concentrations and two dilutions of the plasma are used as in four point assay by Randle (1954c); or several concentrations of insulin and two or more dilutions of the plasma are used (our unpublished data).

Nature vf the substance in the human plasma responsible for its insulin-like activity: Insulin increases the glucose uptake of the rat diaphragm (Stadie and Zapp, 1947; Park and Krahl, 1947; Krahl and Cori, 1947; Mackler and Guest 1953b). Normal human plasma also increases the glucose uptake of the rat diaphragm (Groen *et al*, 1952; Randle, 1954; Vallance-Owen and Hurlock, 1954; and others). Yet the nature of the substance in the plasma responsible for its insulin-like activity cannot be said to be finally established. It will be evident from the following discussion that there are several substances in the plasma which have insulin-like action on the isolated rat diaphragm. There is also convincing evidence that human plasma and especially that of the insulin resistant diabetics contains substances which antagonise the action of insulin. This makes the method less specific for estimating insulin in the plasma. We are not sure as to whether all the increase in the glucose uptake produced by the plasma is due to insulin alone and as to whether all the insulin in the plasma has produced this increase in the glucose uptake.

The factors in the plasma showing insulin-like activity: Millman and Russel (1950) observed a fall in the blood sugar of normal rats after intravenous injection of growth hormone. Park *et al* (1952) noted that injection of pituitary extract in the normal rats caused hypoglycemia in first three hours and during this time the glucose uptake of their diaphragms was increased. Westermyer and Raben (1954) observed the same in mice. Randle (1954a) estimated high plasma insulin values in the patients with acromegaly. He interpretes his finding as showing the insulin-like activity of the excess of growth hormone in the plasma of such patients. We are critical of this interpretation. It may well be that excess insulin is released in the plasma of these patients in order to maintain normoglycemia. The present evidence suggests that the growth hormone of the anterior pituitary causes hypoglycemia in intact animals and increase in the glucose uptake of the diaphragms of treated animals by increasing the insulin secretion. Millman and Russel (1950) noted that injection of growth hormone in the rats with impaired pancreatic function caused a rise in their blood sugar instead of the usual fall. Millman, *et al* (1951) concluded that much of the diabetogenic effect of growth hormone occurs in tissues and increased secretion of insulin presumably occurs in response to growth hormone in normal animals. Campbell *et al* (1954) summarise the position in the following words "Thus there are reasons for the deduction that in normal animals the growth hormone creates in the extrapancreatic tissues an increased demand for insulin and elicits an increased rate of production of insulin from

the beta cells of the islets of Langerhans. This postulated stimulatory effect of growth hormone on the islets is indirect". De Jongh (1954) arrived at a conclusion "Growth hormone secures equilibrium in two different ways.... it increases the quantity of circulating insulin and, in addition, neutralises the effect of insulin on the blood sugar level. Carbohydrate metabolism may proceed unchanged at a raised *insulin* level". Randle (1956c) also thinks that growth hormone may cause insulin realease from the pancreas. Batts *et al* (1956) observed that growth hormone caused increase in the size of the islet tissue of the partially pancreatectomised dogs. Thus the growth hormone is unlikely to have insulin-like action on the tissues. This point has been elaborated here at length as the growth hormone has a more important anti-insulin action on the glucose uptake of the rat diaphragm which will be discussed later.

Park in 1952 attributed the insulin-like activity of the plasma to the nonspecific effect of plasma proteins. He observed that bovine albumen could increase the glucose uptake of the rat diaphragm. Randle (1957) failed to observe any effect of protein on the glucose uptake of the rat diaphragm and argued that the protein used by Park may not have been entirely free from insulin.

Aiman and Kulkarni (1957) observed increase in the insulin effect of the plasma after administration of Carbutamide to normal human volunteers. Only a special design of their experiment could show that it did not indicate an increase in the plasma insulin level.

On the other hand there is considerable evidence to suggest that the normal plasma effect is in fact due to insulin itself. Groen *et al* (1952) demonstrated that the plasma effect was abolished on pancreatectomy in dogs. Randle (1954c) has shown that incubation of the plasma with cysteine destroyed its insulin-like activity. Cysteine is known to destroy insulin. When soluble insulin was injected intravenously in normal subjects prior to withdrawal of blood, the insulin-like effect of the plasma was considerably increased. Insulin effect of the plasma of normal persons increased 90 minutes after ingestion of 50 Gm. glucose (Vallance-Owen *et al*, 1955). Ingestion of glucose is known to produce release of insulin in blood (De Jongh, 1954). High insulin content of the plasma of one patient each with hypoglycemia later proved to be due to beta cell tumour of the islet tissue, was reported by Groen *et al* (1952) and Vallance-Owen and Hurlock (1955). After tentatively accepting the proposition that the plasma effect is due to insulin we now turn to the more disturbing anti-insulin activity of the plasma.

Insulin antagonising substances in the plasma : Himsworth (1949) from the insulin sensitivity tests, classified younger diabetics as insulin sensitive and the

older diabetics as insulin resistant. Bornstein and Lawrence (1951a and 1951b), using ADHA rats inferred that the younger diabetics had no insulin in their plasma whereas the older diabatics had it. This concept of insulin resistance has now changed consequent on the exhibition of insulin antagonising property of the plasma of certain diabetics, usually in the younger age group. Bornstein and Trewhella (1951) demonstrated that the injection of the serum of insulin resistant patients into an ADHA rat prevented the usual fall in the blood sugar after insulin injection. Vallance-Owen *et al* (1955) noted that in some diabetics the plasma insulin activity was nil and if insulin was added to such plasma it was prevented from having its usual effect on the rat diaphragm. Baird and Bornstein (1957) have demonstratated that the plasma of the insulin resistant diabetics actively depressed the glucose uptake of the rat diaphragm. Aiman and Kulkarni (1958) also obtained active depression of the glucose uptake of the rat diaphragm when a 1 in 2 dilution of the plasma of two young diabetics was used. This clearly indicates that the plasma of certain diabetics contains insulin antagonising substances the ones suggested being growth hormone, adrenal cortical steroids, glucagon, adrenaline, and acetoacetates which are normally present in the plasma.

The insulin antagonising action of growth hormone is generally accepted. It was discovered by Young (1937) that fresh saline extracts of the anterior pituitary when given for a sufficiently long time caused diabetes in dogs. Park and Krahl (1949) demonstrated that if anterior pituitary extract was injected into the rats three hours prior to sacrifice the glucose uptake of the rat diaphragm was depressed. Growth hormone produced diabetes in dogs (Houssay and Anderson, 1949, Campbell *et al*, 1950;) and Cats (Cotes *et al*, 1949) and with continued administration of growth hormone in dog insulin resistance develops (De Bodo *et al*, 1950). Ottaway and Smith (1948) and Ottaway (1951) observed that anterior pituitary extract and growth hormone abolished the insulin effect on the rat diaphragm. Hypophysectomy increases insulin sensitivity in rats (Krahl and Park, 1948) and in dogs (De Bodo *et al*, 1950; Sinkoff *et al*, 1954). Hypophysectomy increases the glucose uptake of the rat diaphragm (Krahl and Park, 1948). Injection of anterior pituitary extract or growth hormone in such an animal restores the glucose uptake to the normal level (Park and Krahl, 1949). Whitney and Young (1957b) have shown that in the plasma of the diabetic rats an inhibitor of glucose uptake is present. This inhibitor can appear in the plasma of normal rats if they are given growth hormone and cortisone. Bornstein (1953) thought that the insulin antagonising property of growth hormone which appears some time after injection may be due to some changed product of growth hormone. He substantiated his view by showing that the serum lipoprotein fraction of the injected animals actively depressed the glucose uptake of the rat diaphragm. The precise mechanism of antagonism between

growth hormone and insulin is not clearly understood. The probable significance of this antagonism has been discussed by De Jongh (1954). Therefore in the light of the present knowledge and the state of purification of the hormone we may assume that the diabetogenic and the somatotrophic activities reside in the same fraction (Reid & Young 1948; Reid 1956) though Raben and Westermyer (1952) reported that diabetes could not be produced with their fraction of anterior pituitary which was growth promoting in rats.

Adrenal cortical hormones have an adverse effect on the carbohydrate metabolism of the rat diaphragm. Bartlett *et al* (1949) have shown that DCA and Cortisone inhibit the glucose uptake of the rat diaphragm. Leupin and Verzer (1950) demonstrated that DCA was a more potent inhibitor than cortisone. Verzer (1954) has shown that the cortical steroids diminish the glycogen synthesis of the rat diaphragm. However, apart from this direct action there is a more important effect of facilitating the inhibitory action of the growth hormone. Synergism between the adrenocorticotrophic and the somatotrophic fractions of the anterior pituitary has been adequately demonstrated. In rats growth hormone does not inhibit insulin action in the complete absence of adrenal cortical hormones (Krahl and Cori, 1947; Stadie and Zapp, 1947; Park and Krahl, 1949; Spirtos and Halmi, 1956). In adrenalectomised animals a small dose of cortisone restores the anti-insulin activity of growth hormone. Reid (1951) has shown that ACTH potentiates the diabetogenic action of growth hormone in cats. Locket *et al* (1953) could not produce metahypophyseal diabetes in adrenalectomised cats. In dogs the evidence is not conclusive. Locket *et al* (1953) could make the adrenalectomised dogs given a small daily dose of cortisone, diabetic by the administration of growth hormone. Sinkoff *et al* (1954) noted that the effects of growth hormone were less marked in adrenalectomised dogs maintained on a small dose of DCA. They however felt that growth hormone could produce its effects in absence of adrenal corticoids. But it can be argued that only a small amount of cortical hormones may be needed for the facilitation of the diabetogenic action of growth hormone. Long (1954) maintains that the full activity of the pituitary diabetogenic factor is not manifested unless a certain minimal quantity of adrenal cortical hormone is present. Thus, excess of adrenal cortical hormone in the plasma might antagonise the action of insulin in the plasma and interfere with its assay.

Glucagon, the alpha call secretion (Sutherland and De Duve, 1948) of the pancreas, has been shown by Candella and Candella (1956) to have insulin antagonising action on the rat diaphragm. Randle (1956a) has denied this action as he could not obtain such an effect. Much work yet needs to be done on glucagon to establish its role in normal and abnormal carbohydrate metabolism. The concentration of this hormone is greatest in the pancreatic vein (Bornstein 1951) and it is destroyed in the

liver. Hence the concentration of this substance in the peripheral blood should be very small. It is therefore possible that this substance does not interfere with the plasma insulin estimation by the rat diaphragm method.

Likewise, through, Tuerkischer and Wertheimer (1948) and Walaas and Wallaas (1950) noted that adrenaline depressed the glycogen synthesis of the rat diaphragm and Cohen and Needham (1948) observed an indirect inhibitory effect of adrenaline on the glucose uptake of the rat diaphragm, it is unlikely to be a factor which interferes with plasma insulin estimation by this method.

Stadie and Zapp (1947) obtained a rough quantitative relationship between the insulin concentration and the glycogen synthesis of the isolated rat diaphragm. However it was interesting to note that insulin in a concentration of 5 units per ml. depressed the glycogen synthesis. It cannot be said whether that was the toxic effect of insulin or any other contributory factor which caused this depression.

De Phillips and Iannoccone (1952) thought that the insulin resistance may be due to antibody formation. They found that gamma globulins isolated from the serum of an insulin resistance patient protected rats against the hypoglycemic effect of insulin. Spoont and Dyer (1951) presented a case of insulin resistance with local and general allergy to insulin. It is yet to be adequately proved that antibodies are formed against insulin and real allergy to insulin exists.

Prolactin has also been credited with mild diabetogenic action (Houssay *et al*, 1955).

Apart from these hormones acetoacetates are important substances in the diabetic plasma. Groen *et al* (1952) did not obtain any inhibitory effect on the glucose uptake of the rat diaphragm when ketone bodies were present in the medium in the concentration of 0.3 per cent (the concentration in which they are present in diabetic coma). Chari and Wertheimer (1953) obtained inhibition of glycogen synthesis with a concentration of 0.1 per cent of acetoacetates in the medium. But the glucose uptake was increased. If the glucose concentration in the medium is increased this effect was abolished (Chari and Wertheimer, 1954). Parnes and Wertheimer (1950a and 1950b) have obtained similar findings. Hansen and Rutter (1952) have demonstrated that acetoacetates increased the oxygen consumption of the rat diaphragm. Previous state of nutrition of the animals affects the response of their diaphragm to the acetates (Garner and Roberts, 1954; Parnes and Wertheimer 1950b). Acetate and pyruvate metabolism of the isolated diaphragm has been extensively studied (Villee and Hastings, 1949; Parnes and Wertheimer, 1950a and 1950b; Villee, *et al*, 1952; Foster and Villee, 1954; Chari and Wertheimer, 1953 and 1954). The evidence is not conclu-

sive as to whether acetoacetates affect the glucose uptake in either way. The relative concentration of the acetoacetates seems to be important. Since the concentration of glucose in the medium is kept constant and the acetoacetate concentration in the plasma is variable their relative concentration will vary in each estimation. There is evidence to suggest that insulin resistance, as tested on the isolated rat diaphragm, develops during diabetic acidosis and this resistance disappears during recovery (Fields and Stetten, 1956).

Effective and absolute insulin concentration: from the foregoing it is clear that the glucose uptake of the rat diaphragm may be affected by various factors, other than insulin, present in the normal or the diabetic plasma. The effect produced by the plasma will be the sum total of the effects of insulin and its antagonists in the plasma. We cannot rule out the possibility that some substance in the plasma may have an insulin-like action on the rat diaphragm. As the insulin concentration is calculated from the plasma effect which is the sum total of the above effects Vallance-Owen and Hurlock (1954) termed it as effective insulin concentration of the plasma. Since the blood sugar at any given time must be a function of the effective insulin concentration (Vallance-Owen and Hurlock 1954), estimation of effective insulin concentration does not afford any more help than the estimation of blood sugar. What we really want to know in order to settle the aetiology of diabetes mellitus is the absolute insulin concentration and the relative preponderence of its antagonists. It would be a further advance if we could also determine the nature of insulin antagonising factors in any given case. At present many modifications of the rat diaphragm method are being tried to make it suitable for the estimation of the absolute plasma insulin concentration.

Plasma insulin estimation: Since Groen *et al* (1952) first reported success at the estimation of plasma insulin, many workers (Randle, 1954a,b,c, 1956b, 1957; Vallance-Owen and Hurlock, 1954; Vallance-Owen *et al* 1954, 1955; Wright 1957; Baird and Bornstein, 1957; Aiman and Kulkarni, 1958) have devoted their attention to the problem of plasma insulin estimation. The following table gives the details of the plasma insulin values obtained by various workers.

From the table it is obvious that the variation in the plasma insulin values reported by various workers is so great that any comparison is impossible. The methods differed in the details. However, Wright repeated the work of Vallance-Owen and Hurlock and obtained similar results. This suggests that if a given method is strictly followed the results could be comparable. Unfortunately at present we do not know any method which will measure the absolute insulin concentration of the plasma. Takeuchi *et al*, (1957) reported that dilution of plasma yielded higher insulin values. They could estimate insulin in alloxan diabetic dog plasma when it was diluted 20 times.

TABLE

Normal plasma insulin concentrations as reported by various workers.

Workers	Medium and plasma dilution	Normal insulin concentration microunits/ml.
Groen et al (1952)	Gey and Gey's medium 1 : 5	62·5 to 625
Randle P. J. (1954)	Gay and Gey's medium 1 : 4	9000 to 22000
Vallance-Owen and Hurlock (1954)	Gey and Gey's medium Undiluted Plasma	40 to 80
Baird and Bornstein (1957)	Extract of the plasma	1000 to 2000
Peter Wright (1957)	Gey and Gey's medium Undiluted plasma	40 to 80
Aiman and Kulkarni (1958)	Stadies medium 1 : 10 dilution	700 to 6300

Undiluted plasma of these animals had actively depressed the glucose uptake of the rat diaphragm. They assumed that dilution of the plasma diluted the anti-insulin factors to the level of ineffectivity while the insulin remained effective. Thus they could estimate the absolute insulin concentration. Wright (1957) also noted that dilution of the plasma yielded higher insulin values. He also considers the possibility that the bound insulin might get released on dilution. Randle (1957) observed that fourfold dilution of insulin diminished the insulin effect whereas fourfold dilution of plasma did not decrease the plasma effect. In two juvenile diabetics in our series (Aiman and Kulkarni, 1958) where a 1 in 2 dilution of the plasma actively depressed the glucose uptake of the rat diaphragm a 1 in 10 dilution showed insulin activity in the plasma. This dilution technic gave us a large scatter in the values of normal plasma insulin. We do not know how far the dilution affects insulin and its antagonists. The relative influence of anti-insulin factors must vary at different periods. It must also be different in different individuals. Therefore the critical dilution must be individualised. This would make the method more complicated with a doubtful prospect of estimating absolute insulin concentration.

Baird and Bornstein (1957) used an elaborate process to extract insulin in the plasma which would be free from the anti-insulin factors. Their data

is small and it is yet to be adequately proved that the whole of the insulin in the plasma is obtained in the extract and that such an extract is free from the factors which interfere with the glucose uptake of the rat diaphragm. If a suitable extraction method is established it may solve the problem of absolute insulin estimation.

Obviously then the rat diaphragm method is not very specific for insulin in the plasma. The sensitivity of the method is sufficient to be able to estimate insulin in the concentration in which it is present in the plasma. We will have to try and establish such modification of the method as will enable us to estimate absolute insulin concentration and the anti-insulin factors. In our effort to estimate insulin in the plasma we should not lose sight of the other methods such as glucose utilisation of the isolated mammalian heart (Bleehan and Fisher, 1954) and gas output of the lactating mammary gland (Balmain *et al*, 1954).

While a specific quantitative chemical method is always better than a biological one—paper chromatographic separation (Robinson and Kitty, 1952; Porter, 1953) if successful might solve a great problem—amongst the biological methods, the rat diaphragm method, at present holds promise of being established for plasma insulin estimation.

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