Determination of Serum Insulin by the Rat Diaphragm Method

Further Observations in Diabetic and Nondiabetic Subjects and in Hyperinsulinism

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In 1952 Groen et al.[1] described a method for the determination of the insulin content of blood serum with the aid of the isolated rat diaphragm. Since our last publication,[2] a few papers of other authors dealing with the same subject have been published. It is the object of this communication to review this work and to describe our further experiences.

Using a technic with a singled hemidiaphragm for each determination, Vallance-Owen and Hurlock[3, 4] confirmed the fundamental observation that normal serum increases the glucose uptake of diaphragm tissue. This increase is inhibited by incubation of the serum with cysteine or glutathione, which makes it probable that it is due to insulin. With their technic these authors calculated the normal fasting level of plasma insulin to be between 3 and 8 x 10^{-5} U/ml. This is much less than the figures reported by Groen et al. One hour after 50 gm. of glucose by mouth they found up to tenfold increases in insulin content of the serum over the fasting level.[3]

Randle[5] likewise demonstrated the effect of normal serum with the aid of the rat diaphragm test and confirmed that this effect diminished or disappeared after treating the serum with cysteine or glutathione. Small amounts of insulin were recovered satisfactorily when added to normal serum. This author found the insulin content of normal human plasma, taken two and a half hours after 50 gm. of glucose by mouth, to be 10-20 x 10^{-3} U/ml. These figures are higher than those given by Vallance-Owen et al. and by Groen et al.[1] for fasting values. The insulin activity of plasma from acromegalic patients was found by Randle to be significantly greater,[6] the activity of plasma obtained from patients with hypopituitarism[7] significantly less than that of normal human plasma. On this evidence the author suggested that the level of growth-hormone might increase or influence the insulin content of the insulin-like activity of human plasma.

These results, which to a great extent confirmed our findings, have encouraged us to use the rat diaphragm method for a more systematic investigation of the insulin content of the blood in health and disease.

METHODOLOGY

The determinations of blood insulin in the present investigation were performed as described previously[1] with the following modifications:

1. In most cases the whole diaphragms of eight rats of 80-100 gm., fasted for twenty-four hours, were divided into five pieces in order to be able to compare two unknown solutions with two insulin solutions of known strength (usually 10^{-4} and 10^{-3} U/ml.). As the sensitivity of the diaphragm for insulin diminishes by dividing the tissue in smaller pieces, the insulin effect (for the same insulin concentration) is smaller with fifth diaphragms than with quarter diaphragms (as used previously). However, the difference is not great and therefore this disadvantage was accepted with the advantage of testing two unknown solutions in one experiment.

In every determination this procedure of pooling the fifth diaphragms of eight rats was repeated five times, so that a statistical calculation of the significance of the results was possible.

2. The pooled fifth diaphragms were weighed prior to the incubation in the glucose buffer serum-mixtures instead of afterwards.

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3. Glucose determinations were carried out with the anthrone method described by Van Munster[8] in the following way: 0.5 ml. of incubation fluid is deproteinized with 5 ml. of 5 per cent trichloroacetic acid. After centrifuging 0.5 ml. of the clear supernatant solution is mixed in a test tube with 10 ml. of a solution of 0.2 per cent anthrone in sulfuric acid of specific gravity 1.72 and then heated in a waterbath at 100° C. for six minutes. After cooling the light absorption of the resulting blue-green solution is measured at 635 mμ, and the observed extinction values compared with the extinction values of known glucose solutions treated in the same way.

4. Serum dilutions in the glucose buffer mixture were always 1 in 5 unless otherwise stated.

5. From the figures obtained for the increase in glucose utilization by the addition of the serum, the insulin concentrations in the serum solutions were calculated in a way similar to that described by Randle.[5]

This author showed that a linear standard curve is obtained when the cube root of the absolute glucose uptake is plotted against the logarithm of the insulin concentration. As can be calculated from the data given by Randle in his publication, a linear regression line is also obtained when the cube root of the insulin effect on the glucose utilization is used instead of the glucose uptake itself. We could confirm this experimentally (figure 1), at least approximately, when working with insulin concentrations ranging from 10^{-4} to 10^{-2} U/ml. of incubation medium.[9]

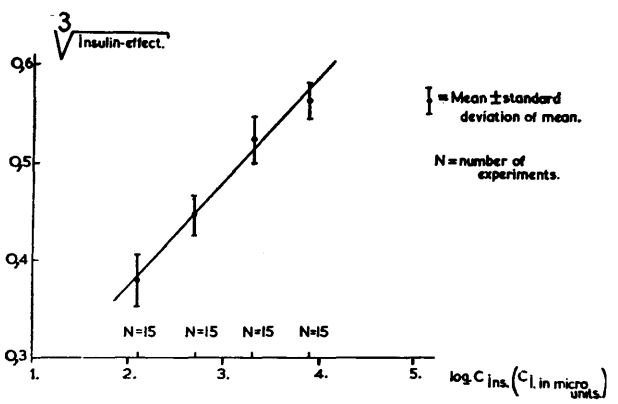

FIG. 1. Regression line showing linear relationship between the logarithm of the insulin concentration (in micro units per ml. of incubation medium) and the cube root of the effect of insulin on glucose utilization (mg. of glucose per 100 mg. of wet tissue).

In every determination the effects obtained with two insulin concentrations of known strength were used to construct a linear regression line in the way outlined above and with the aid of this regression line the insulin content of the serum under investigation was calculated.

SENSITIVITY AND ACCURACY OF THE RAT DIAPHRAGM METHOD

All investigators, who work with the rat diaphragm as a test-preparation for small concentrations of insulin, report that the sensitivity of the preparation can vary to a fairly large extent, even when a rigidly standardized technic is used. The cause of these variations is still unknown.

The lower limit of sensitivity in the test as reported in the literature differs rather from author to author. In recent publications Vallance-Owen et al.[3] report that 10^{-5} U/ml. could still be detected while Randle[5] found that 10^{-4} U/ml. is the lowest concentration of insulin that produces a significant stimulation of glucose uptake. In our former papers, [1, 10] we reported that the lower limit of sensitivity found with the technic described there was often 10^{-5} U/ml., especially when working with hemidiaphragms. Since then, especially when fifth diaphragms are used, the minimum concentration exerting an effect was found to be higher, viz 10^{-4} U/ml.

The variations in the sensitivity of the diaphragm tissue for insulin, from experiment to experiment, make it necessary to run standard insulin solutions together with the solutions to be tested in every experiment.

To give an impression of these variations in sensitivity and also of the reliability of values obtained for the effects of insulin, we have put together in table I the results obtained with two insulin concentrations of known strength (10^{-4} and 10^{-3} U/ml.) in forty-eight experiments. Every experiment consisted of five replicates. The mean value of the effect observed with addition of 10^{-4} U/ml. in such an experiment was designated as *a*; with 10^{-3} U/ml. as *b*; the standard deviations respectively as sdp_a and sdp_b; all values expressed as mg. of glucose/100 mg. wet tissue in ninety minutes.

Of these forty-eight experiments eight had to be discarded because *a* and/or *b* did not differ significantly from zero or because there was no significant difference between *a* and *b*. In one experiment *a* and *b* were abnormally high, viz three times the mean value found in the remaining thirty-nine experiments. Of these remaining experiments the mean value of *a* was 0.076 ± 0.033 and of *b* more than twice as high, viz 0.175 ± 0.052 mg. of glucose per 100 mg. of rat tissue in ninety minutes. The mean value of the standard deviation sdp_a was 0.036 and of sdp_b 0.051. The

TABLE 1

Variation in sensitivity of the rat diaphragm for insulin in 48 experiments. Every experiment consisted of five replicates

	Number	Effect of pure insulin upon glucose uptake (mg. glucose/100 mg. wet tissue/90 min.) observed from: 10^{-4} U/ml.	10^{-3} U/ml.
Total number of experiments	48		
Discarded on account of insignificant effects of pure insulin	8		
Abnormal high effects of pure insulin	1	$a=0.233\pm0.065$	$b=0.411\pm0.79$
Remaining experiments: mean value of a, resp. b $\pm$ stand.-deviation	39	$a=0.076\pm0.033$	$b=0.175\pm0.052$
Mean value of sdp_a, resp. and sdp_b	39	$sdp_a=0.036$	$sdp_b=0.051$
Mean value of slope of regression line	39	1.38 ± 0.44 (n=39)	

$a\pm sdp_a$ = mean value $\pm$ standard deviation of effect of 10^{-4} U/ml. in one experiment.
$b\pm sdp_b$ = mean value $\pm$ standard deviation of effect of 10^{-3} U/ml. in one experiment.

slopes of the regression lines have been calculated also from these experiments; the mean value was 1.38 $\pm$ 0.44.

As a result of the rather large standard deviations of the effects of insulin and of serum on glucose uptake and because the relationship between the effect observed and the concentration of insulin to be determined is a logarithmical one, the reliability of the estimation of serum insulin is rather small.

Randle showed that in his experiments and using his method of calculation the limits of error (at the 5 per cent level of probability) for the calculated value of serum insulin were about one-third of and three times the value found. This means that for a calculated serum insulin concentration of 3 mU/ml. the limits of error are 1 and 9 mU/ml. In our experiments the limits of error have not been calculated but are very probably of the same order.

INSULIN ACTIVITY OF SERUM IN HEALTH AND DISEASE

In our previous papers only a few figures for the insulin activity of human serum from healthy persons and patients were given. In the last three years we have tested some fifty sera with the technic described above. Calculations were made as described and the insulin activity expressed as milli-units of insulin per ml. of serum. The results are collected in figure 2.

Normal human serum. Fourteen samples of serum have been tested. Twelve of them were obtained from patients who did not suffer from any disease interfering with carbohydrate metabolism. Their blood was taken one or two days before they left the hospital after recovery. One sample was obtained from one of the investigators and one from a healthy child of three years old. The blood was taken fasting and in the

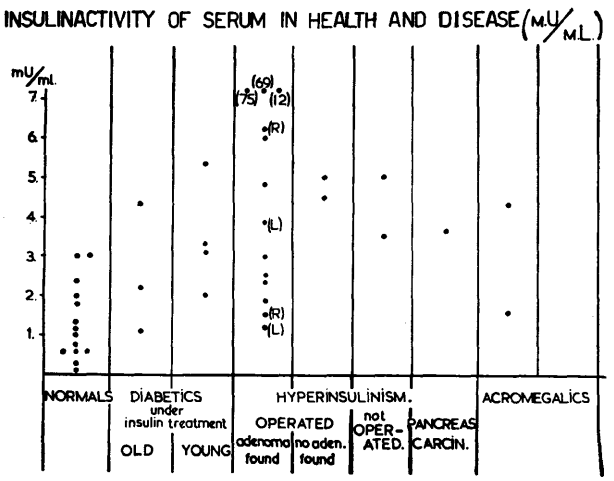

FIG. 2. Serum insulin activity in health and disease, expressed as milliunits (=10^{-3} U) of insulin per ml. of serum determined by the rat diaphragm method.

resting state. After clotting in the icebox the blood was centrifuged rapidly and the serum kept at — 4° C. till the assay. The values found for these normal samples (figure 2, column 1) range from 0.1 to 3.0 milli-units of insulin per ml. of serum. The range of normal figures is therefore higher than recorded previously by us. These normal values are also considerably higher than those given by Vallance-Owen et al., who found always less than 0.1 milli-units of insulin per ml. of plasma in the fasting state. Randle on the other hand reported values ranging from 10-20 mU per ml. in the plasma of healthy individuals taken two and a half hours after they had received 50 gm. of glucose by mouth under normal working conditions.

It is not clear how these great discrepancies in normal values must be explained. Randle and Vallance-Owen et al. use plasma for the determination while we use serum. This however does not seem of great im-

portance in this connection, for it was found by Randle[11] as well as by ourselves[12] that serum and plasma, taken at the same time from the same patient, showed no significant difference in insulin activity. A factor of perhaps more importance may be the effect of the glucose given by Randle two and a half hours before blood was drawn. Vallance-Owen reported an almost tenfold increase in plasma insulin one hour after 50 gm. of glucose orally. We are at present investigating this possibility, which might be of some importance, but it does not seem probable that it gives the explanation of the above mentioned discrepancies. The possibility remains that the differences are due to small differences in technic between the laboratories. To test this a sample of lyophylized normal human plasma prepared by Randle was assayed for insulin activity by Randle in Cambridge and by us in Amsterdam. Randle found 3.2 mU and we 2.6 mU/ml. of original plasma, figures that agree very well. In a lyophylized sample of plasma taken from an acromegalic patient, however, we found only 4.4 mU/ml. whereas Randle found 121 mU/ml.

It can be said, therefore, that the reason why the insulin values found in human plasma with the aid of the test by different authors vary so widely is still unknown.

Diabetics under insulin treatment. We examined the serum from seven patients suffering from diabetes under insulin therapy. Three of them were elderly women without ketosis who took only moderate amounts of insulin. The four others were young people who developed ketosis rapidly when insulin therapy was interrupted and who required more insulin to control the disease. The blood of all these patients was taken 12 to 18 hours after the last insulin injection. In column 2, figure 2, are given the values found in the old and in column 3 the values found in the young patients. All values were well above 1 mU/ml., ranging from 1.1 to 5.2 mU/ml. of serum.

From these figures it is not possible to conclude that there is a significant difference between the serum insulin content of these two groups of diabetics, at least when they are both receiving insulin.

However Bornstein and Lawrence,[13] using hypophysectomized-alloxan diabetic-adrenalectomized rats as test animals, and more recently Vallance-Owen, Hurlock and Pease,[4] using the rat diaphragm test, reported that they have found differences in serum insulin. The latter authors observed that in their diabetics who did not require insulin (most of them older obese women who did not develop ketosis rapidly), plasma insulin activity was as high or even higher than in normal individuals. On the other hand the plasma of most, but not of all, insulin-requiring diabetics did not stimulate the glucose utilization of the rat diaphragm, especially not when the blood sugar level was well outside the physiological range. Neither was any insulin activity detectable when small amounts of pure insulin were added in vitro to the plasma of these patients. The activity of added insulin appeared to be inhibited by the plasma in these cases.

Up till now we have not been able to confirm these observations; more especially we have found, at least in the rat diaphragm test, no evidence for the presence of an insulin inhibitor in the serum of diabetic patients, even if the blood was taken during coma.

Hyperinsulinism

During the last three years we had the opportunity to examine the serum of some fifteen patients who were suspected of suffering from hyperinsulinism. All patients had hypoglycemic attacks especially when fasting. Many of them had mental disturbances during the attacks. The serum insulin determinations were carried out on blood taken during hypoglycemic attacks, when possible.

Thirteen of these patients were operated. In eleven of these thirteen cases the diagnosis of islet adenoma of the pancreas was confirmed; in some instances rather large adenomas could be removed, in other cases partial or complete pancreatectomy was performed and histological examination of the resected part of the pancreas revealed adenomatous tissue. The results of the tests on insulin activity performed with the serum of these eleven patients before the operation are given in figure 2, column 4. Seven of these values are definitely above the highest one found for normal serum (3 mU/ml.). The four others overlapped with the upper normal range.

In two operated cases no adenomatous tissue was found in the part of the pancreas that had been removed, though the condition of these patients improved after the operation. The serum insulin activity in these two cases was increased, viz 5 and 4.5 mU/ml. (figure 2, column 6).

The two remaining patients were children. They have not been operated so far. The insulin activity of their sera was 3.4 and 5.0 mU/ml., respectively (see figure 2, column 6). We examined the blood serum of one patient with a carcinoma of the islets of the pancreas who also suffered from hypoglycemic attacks. His blood serum insulin activity was found to be 3.6 mU per ml. (figure 2, column 6).

These findings with the serum of patients suspected to suffer from hyperinsulinism show that the determi-

nation of serum insulin may be of value in the diagnosis of this disease. In four cases the insulin activity of the serum sample was within the upper normal range. It should be mentioned, however, that in two of these cases (column 4, L. and R.) the insulin content of two serum samples was determined. One of these showed a normal, the other a high value. It cannot be excluded that this discrepancy was due to the limited accuracy of the procedure, but it is also possible that the insulin activity of serum of patients with hyperinsulinism varies from day to day, so that it may be necessary to perform the determinations on more than one occasion.

SUMMARY

The insulin activity of blood serum in health and disease can be determined in vitro by the rat diaphragm method as described previously. The normal fasting values range from 0.1-3 milli-units per ml. of serum. In diabetics under insulin treatment the serum insulin values were found to be normal or slightly elevated. In 9 of 11 cases of hyperinsulinism significantly increased insulin activity was found.

The method, although apparently able to detect gross changes in insulin content, as occurring in pathological conditions, is still insufficiently accurate for the study of smaller fluctuations in plasma insulin activity under physiological circumstances. Most disturbing is the fact that the values, reported by different investigators for the normal serum insulin activity, vary widely. The elucidation of the cause of this variation may lead to further improvements of the method.

SUMMARIO IN INTERLINGUA

Determination del Nivello Seral de Insulina per Medio del Methodo de Diaphragma de Rattos

Le activitate insulinic de sero sanguinee in sanitate e morbo pote esser determinate in vitro per le previemente describite methodo a diaphragma de rattos. Le normal valores in stato jejun varia ab 0,1 a 3,0 milli-unitates per ml de sero. In diabeticos sub tractamento insulinic, le valores del insulina seral se monstrava normal o levemente elevate. In 9 ex 11 casos de hyperinsulinismo, significative augmentos del activitate de insulina esseva constatate.

Le methodo, ben que apparentemente capace a deteger grossier alterationes del contento de insulina occurrente in conditiones pathologic, es ancora insufficientemente accurate pro le studio de minor fluctuationes del activitate de insulina in le plasma sub conditiones physiologic. Le plus disquietante facto es que le valores reportate per varie investigatores pro le normal activitate de insulina seral exhibi grande variationes. Le clarification del causas de iste variationes va possibilemente resultar in meliorationes additional del methodo.

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