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ASSAY OF PLASMA INSULIN ACTIVITY BY THE RAT-DIAPHRAGM METHOD

BY

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Bornstein (1950) described a method for the assay of insulin in human plasma which was based on the fall of blood sugar of alloxan-diabetic hypophysectomized adrenalectomized rats (A.D.H.A. rats). Subsequently Bornstein and his colleagues determined the insulin content of plasma from various types of diabetic by this method (Bornstein and Trewhella, 1951; Bornstein and Lawrence, 1951a, 1951b). The published results of these workers were of such interest as to merit further study.

A.D.H.A. rats prepared in this laboratory have proved to be too delicate and to possess too unstable a blood sugar to permit their use for accurate insulin assay. Groen *et al.* (1952) described a method for the detection and assay of insulin in human plasma which was based on the glucose uptake of the isolated normal rat diaphragm surviving *in vitro*; as was first shown by Gemmill (1940), this uptake is raised by the *in vitro* addition of insulin to the fluid in which the isolated diaphragm is suspended. This method has been critically investigated as a quantitative procedure for the assay of insulin and a modified procedure applied to the determination of the insulin activity of normal human plasma.

Methods

Animals.—The hemidiaphragms of normal male albino Wistar rats of 100–150 g. were used in these experiments. The animals were starved for 18 to 24 hours before use.

For the preparation of alloxan-diabetic hypophysectomized rats (A.D.H. rats) male albino Wistar rats of 170–220 g. were used. They were first of all hypophysectomized, and then, one week later, made diabetic by the intravenous administration of alloxan.

Insulin.—A single sample of crystalline insulin (Boots) of potency 23.2 units per mg. was used as standard in these experiments. Solutions of this were freshly prepared for each assay.

Human Subjects.—The different samples of plasma collected from human subjects are designated as ran-

dom (collected without previous preparation of the subject) or fed (collected from subjects two and a half hours after the oral administration of 50 g. of glucose).

Chemical Analysis.—Blood glucose and the glucose content of the incubation fluid in the insulin assay was determined colorimetrically by the method of Somogyi (1945).

Procedure

For the qualitative detection of insulin activity in human plasma the glucose uptakes of rat hemidiaphragms suspended in buffer were compared with the glucose uptakes of rat hemidiaphragms suspended in buffer to which the plasma had been added.

For the quantitative assay of insulin activity in human plasma two methods have been used, the single standard method and a four-point assay.

In the single standard method, the absolute glucose uptake of rat diaphragm in the presence of plasma was compared with the absolute glucose uptake of rat diaphragm in the presence of a single standard dose of insulin (2 milliunits per ml.). In each assay (unless otherwise stated) four hemidiaphragms (from different rats) were used for the determination of the basal glucose uptake and six for the determinations of the glucose uptakes in the presence of plasma and of insulin respectively. A standard dose of insulin of 2 milliunits per ml. was chosen, since this gave results very similar to those yielded by normal human plasma under these conditions.

In four-point assays the glucose uptakes of rat hemidiaphragms in the presence of two dilutions of plasma (1:16 and 1:4) and two concentrations of insulin (0.5 and 2 milliunits per ml.) were simultaneously determined.

Experimental Procedure

The basal glucose uptake of the isolated rat diaphragm was determined in buffer (Gey and Gey, 1936) containing 2.5 mg. per ml. of glucose. The glucose uptake in the presence of insulin was determined in buffer-glucose containing the appropriate concentration of insulin. For the determination of the glucose uptake in the presence of plasma, buffer-plasma mixtures were prepared containing 2.5 mg. of glucose and 0.25 ml. of plasma in each ml. of mixture. In all cases the gas phase during incubation was 93% O₂, 7% CO₂.

In typical experiments, human blood was drawn from the antecubital vein by a dry sterile syringe. The blood was transferred to centrifuge cups containing a little heparin and the plasma separated by centrifugation. The plasma sugar was then determined, and after preparation of the buffer-plasma mixture 1-ml. samples of the incubation fluids were placed in the main compartments of Warburg manometer flasks. Normal rats were then killed by decapitation and their diaphragms removed, divided, and washed for exactly five minutes at room temperature in buffer-glucose (glucose concentration 2.5 mg. per ml.) previously saturated with 93% O₂, 7% CO₂. The hemidiaphragms were then lightly blotted, transferred to the manometer flasks, and incubated for three hours at 37° C. at a shake rate of 100 cycles a minute.

After incubation the flasks were removed from the bath, cooled, and 0.2 ml. of the fluid taken for glucose estimation. The hemidiaphragms were then blotted dry and weighed on a torsion balance.

Calculation of Results

The glucose uptake of the diaphragm was calculated as mg. glucose/g. wet wt. diaphragm/hour. The estimate of variance given in the tables is the standard error of the mean.

In single standard assays the insulin activity of the plasma was calculated from a log-dose (of insulin)/response curve previously determined, the standard dose of insulin incorporated in the assay being used to fix the position of this

*In receipt of a full-time grant from the Medical Research Council.

log-dose response curve. In this calculation the slope of the log-dose response curve was assumed to be constant. The limits of error ($P=0.05$) were calculated from previously determined regression data for crystalline insulin, since the variance in different assays did not differ significantly.

In four-point assays the insulin activity of the plasma and limits of error were calculated from the assay data by the usual means.

The insulin activity of the plasma is expressed as milliunits of insulin per ml. of plasma (1 milliunit is 10^{-3} unit).

Results

Response of Rat Diaphragm to Insulin Added In Vitro

Table I and Fig. 1 record the glucose uptake of rat diaphragm in the presence of different concentrations of insulin added *in vitro*. The results are the mean values of data collected on six separate occasions during a two-weeks period in September, 1953.

FIG. 1.—The effect of increasing concentrations of insulin upon the glucose utilization of the isolated rat diaphragm expressed as a log-dose (of insulin) response curve.

FIG. 2.—Four-point assays of the insulin activity of normal human plasma, by the rat-diaphragm method. Slopes of log-dose response curve for two concentrations of insulin and two dilutions of plasma. Insulin concentrations plotted as log dose (of insulin) in microunits per ml. Plasma insulin activity plotted as insulin concentrations in arbitrary units.

precision ($\lambda=s/b$) was 0.36. The slope of the log-dose (of insulin) response curve has been recently redetermined (January, 1954) and found to be 0.7081, in good agreement with the previous value.

The Insulin Activity of Normal Human Plasma

The method described has been applied to the determination of the insulin activity of plasma collected from normal human volunteers two and a half hours after the oral administration of 50 g. of glucose. The results of similar assays in acromegaly and hypopituitarism have been described in separate papers (Randle, 1954a, 1954b).

TABLE I.—Effect of Increasing Concentrations of Insulin, Added In Vitro, Upon the Glucose Utilization of the Isolated Rat Diaphragm

Insulin Concentration Microunits/ml. (= 10^{-6} unit)	Absolute Glucose Uptake	$\sqrt[3]{(\text{Absolute Glucose uptake} \times 100)}$		No. of Hemi- diaphragms
		Observed	Calculated from Linear Regression	
0	2.32 ± 0.05	—	—	16
125	2.96 ± 0.09	6.665	6.679	16
500	3.55 ± 0.08	7.081	7.090	16
2,000	4.25 ± 0.06	7.518	7.505	16
8,000	5.03 ± 0.09	7.952	7.910	16
32,000	5.73 ± 0.10	8.307	8.321	16

All glucose uptakes with insulin were significantly greater than the basal glucose uptake. Each increase in dose recorded produced a significant increment in response.

The mean plasma insulin activity by the single standard method was 13.5 milliunits per ml. (limits of error 12 to 19.8 milliunits per ml.); by four-point assays the mean plasma insulin activity was 12.4 milliunits per ml. (limits of error 6 to 24 milliunits per ml.). The experimental details and individual results are recorded in Tables II and III and Fig. 2.

TABLE II.—Insulin Activity of Plasma from Normal Human Subjects 2½ Hours After Oral Glucose, as Assayed by the Single Standard Method

Normal Human Subjects (Fed)	Blood Sugar	Glucose Uptake in Presence of		Plasma Insulin mU./ml.	Limits of error $P=0.05$	N
		Plasma Diluted 1:4	Insulin 2 mU./ml.			
1	103	3.95 ± 0.17	3.52 ± 0.23	20	8 to 53	12
2	77	4.18 ± 0.03	4.03 ± 0.08	11	5 to 28	12
3	102	4.14 ± 0.04	4.02 ± 0.27	10	4 to 26	12
4	72	4.16 ± 0.17	4.10 ± 0.14	9	3 to 26	12
5	80	4.27 ± 0.12	3.84 ± 0.22	19	6 to 62	8
6	89	4.65 ± 0.14	4.51 ± 0.14	10	3 to 32	8
7	90	4.26 ± 0.42	3.76 ± 0.11	22	8 to 63	11
Mean:				13.50	12 to 19.8	(75)

N is total number of hemidiaphragms used for determining the mean glucose uptake in the presence of plasma and of insulin.

TABLE III.—Insulin Activity of Plasma from Normal Human Subjects 2½ Hours After Oral Glucose, as Assayed by Four-point Assays

Assay No.	Glucose Uptake with Insulin		Glucose Uptake with Plasma		Plasma Insulin mU./ml	Fiducial Limits P=0.05
	Concentrations		Dilutions			
	0.5 mU./ml.	2 mU./ml.	1 : 16	1 : 4		
1	3.14 ± 0.18	3.84 ± 0.22	3.48 ± 0.19	4.27 ± 0.12	16.7	7 to 38
2	2.93 ± 0.12	4.51 ± 0.14	3.10 ± 0.14	4.65 ± 0.14	10	7 to 14
Mean:					12.4	6 to 24

Four observations at each dose level.

All glucose uptakes significantly greater than the basal glucose uptake.

Recovery of Insulin Added to Plasma In Vitro

In Table IV are recorded the results of experiments in which the insulin activity of insulin-plasma mixtures prepared *in vitro* was assayed. In these experiments insulin

TABLE IV.—Recovery of Insulin Added In Vitro to Buffer-plasma Mixtures Prepared in the Usual Way

Basal Glucose Uptake	Glucose uptake due to plasma with insulin added <i>in vitro</i> to give a total estimated concen- tration of 8.25 mU./ml.	Glucose uptake due to Insulin. Concentration, 8 mU./ml.
2.54 ± 0.18 (4) 2.30 ± 0.05 (4)	5.02 ± 0.27 (6) 4.33 ± 0.21 (6)	5.08 ± 0.27 (6) 4.08 ± 0.10 (6)

Both estimates performed with a single sample of normal human plasma collected 2½ hours after oral administration of 50 g. of glucose. Insulin content, 13 mU./ml.

Figures in parentheses = number of hemidiaphragms.

For first assay, estimated insulin content of insulin-plasma mixture is 7.7 mU./ml. (limits of error, $P=0.05$, 2 to 30 mU./ml.).

For second assay, estimated insulin content of insulin-plasma mixture is 12.6 mU./ml. (limits of error $P=0.05$, 3 to 33 mU./ml.).

Recovery of added insulin not significantly different from 100% in either case.

was added, *in vitro*, to buffer-plasma mixtures prepared in the usual way, to bring the insulin content to a calculated value of approximately 8 milliunits per ml. The glucose uptake of the diaphragm in this mixture was then compared with the glucose uptake of the diaphragm in the presence of insulin at a concentration of 8 milliunits per ml. The recovery of insulin added to plasma *in vitro* and assayed in this way did not differ significantly from 100%.

Insulin Activity of Normal Human Plasma Assayed in the A.D.H. Rat

Previous workers have reported the insulin content of normal human plasma, collected after a glucose meal, to be 0.34 milliunit per ml. (Bornstein and Lawrence, 1951b), 1 milliunit per ml. (Groen and Willebrands, personal communication), and 0.1 to 0.6 milliunit per ml. (Vallance-Owen and Hurlock, 1954). The value obtained by the rat-diaphragm assay described in this paper is considerably greater than these reported values.

The discrepancy between whole-animal methods and the rat-diaphragm technique described in this paper is illustrated by the assay using A.D.H. rats (Table V). In this experiment the change in blood sugar of A.D.H. rats over

TABLE V.—Effects of Intravenously Administered Saline, Normal Human Plasma, and Insulin Upon the Blood Sugar of Alloxan-diabetic Hypophysectomized Rats

Nature of Injected Material	Mean Fall of Blood Sugar $\pm$ S.E. Mean	No. of A.D.H. Rats	t	P
1. Saline (1 ml.)	-4.6 $\pm$ 4.1	8		
2. Normal human plasma* (1 ml.)	-13.8 $\pm$ 8.8	5	—	—
3. 2 milliunits of insulin in 1 ml. of saline	-41.5 $\pm$ 5.2	5	<i>t</i> for 1 v. 3 is 9.3 <i>t</i> for 2 v. 3 is 6.8	< 0.001 < 0.001

* 1 ml. of this plasma possessed activity equal to that of 13 mU. of insulin in the rat-diaphragm assay.

a period of 20 minutes following the intravenous injection of normal human plasma was measured. It was observed that plasma, assayed by the rat-diaphragm method to contain 13 milliunits of insulin per ml., had no effect upon the blood sugar of A.D.H. rats. By contrast 2 milliunits of insulin produced a highly significant fall of blood sugar in the same A.D.H. rats under similar experimental conditions.

Insulin Activity of Cysteine-treated Plasma

Du Vigneaud *et al.* (1931) observed that cysteine treatment inactivated insulin. Other workers who have used the rat-diaphragm assay for insulin have reported that cysteine will abolish the insulin activity of human plasma or serum. This observation has been confirmed in experiments in which normal human plasma was incubated with cysteine (concentration 20 mg. per ml.) for 12 to 18 hours. The experiments were controlled by simultaneous incubation of the same plasma in the absence of cysteine. It was observed that incubation with cysteine consistently abolished the insulin activity of plasma, whereas plasma incubated

without cysteine still possessed significant insulin activity. It was observed in additional experiments that the basal glucose uptake determined in buffer incubated with cysteine was similar to the basal glucose uptake in untreated buffer. The results of typical experiments are recorded in Table VI. Some samples of cysteine-treated plasma were found to inhibit the glucose utilization of rat diaphragm, but this effect was not consistently seen (Table VI).

Samples of cysteine-treated plasma which had been dialysed against buffer for 18 hours, to remove residual cysteine, were also without insulin activity (Table VI). By contrast, untreated human plasma does not lose its insulin activity when dialysed against buffer (Table VI).

Discussion

Assay of Insulin by the Isolated Rat Diaphragm

The rat-diaphragm technique for the assay of insulin has not previously been critically investigated by conventional statistical methods. The results of statistical appraisal of the rat-diaphragm technique, reported in this paper, suggest that it is not a very precise method for the assay of insulin, though the index of precision ($\lambda=0.36$) compares favourably with that of some methods at present used for the bio-assay of other hormones—for example, the tibial band assay for growth hormone. It is difficult to compare the results of this investigation of the rat-diaphragm assay for insulin with those previously reported, since other workers have not supplied comparable statistical data. The variance encountered in the experiments reported in this paper is similar to that found by other workers who have studied the glucose uptake of the isolated rat diaphragm—for example, see Ottaway (1953).

The sensitivity of the isolated rat diaphragm to insulin was found in these experiments to vary considerably from time to time and the variation in sensitivity was not necessarily seasonal; in disagreement with previous reports (*Groen *et al.*, 1952; Vallance-Owen and Hurlock, 1954). The minimum effective concentration of insulin in these experiments was 10^{-4} unit per ml., though some workers have claimed that as little as 5×10^{-6} unit of insulin per ml. will increase the glucose utilization of the isolated rat diaphragm (Groen *et al.*, 1952).

Specificity of the Rat-diaphragm Assay for Insulin in Plasma

Many workers have previously reported that the plasma of normal animals will increase the glucose uptake of rat diaphragm when added to the fluid in which the isolated diaphragm is suspended. This effect has been variously attributed to non-specific effects of plasma protein (Park, 1952), or to insulin in the plasma (Tuerkischer and Wertheimer, 1948; Groen *et al.*, 1952; Vallance-Owen and Hurlock, 1954).

Park attributed the insulin activity of normal rat plasma in the rat-diaphragm assay to non-specific effects of plasma protein, since he found that crystalline bovine serum albumin and A.D.H. rat plasma would stimulate the glucose uptake of the isolated rat diaphragm. However, these experiments do not prove conclusively that the effects of normal plasma are not due to insulin, since crystalline bovine serum albumin and A.D.H. rat plasma may well contain insulin. In this connexion Gemmill (1940) reported that crystalline ovalbumin did not increase the glucose utilization of the isolated rat diaphragm.

Groen *et al.* concluded from their experiments that the insulin-like activity of plasma from normal human subjects and dogs in the rat-diaphragm assay was due to insulin, since the activity was not found in plasma obtained from severely diabetic patients and depancreatized dogs. The validity of this conclusion is, however, doubtful, since Park (1952) and Bornstein (1953) have reported that the plasma of alloxan-diabetic rats contains endocrine factors which inhibit the glucose utilization of the isolated rat diaphragm. The failure of Groen *et al.* to detect insulin-like activity

TABLE VI.—Insulin Activity of Human Plasma After Dialysis and After Treatment with Cysteine as Detected by the Rat-diaphragm Technique

Addition to medium of:	Change in Glucose Utilization	N	t	P
Dialysed normal human plasma	1.07 $\pm$ 0.19	6	3.5	< 0.02 > 0.01
Buffer incubated with cysteine	0.05 $\pm$ 0.09	6	—	—
Normal human plasma (1)				
Incubated with cysteine	-0.57 $\pm$ 0.10	6	4.40	< 0.01
Incubated without cysteine	1.00 $\pm$ 0.12	6	5.50	< 0.01
Normal human plasma (2)				
Incubated with cysteine	0.14 $\pm$ 0.21	6	—	—
Incubated with cysteine and dialysed	-0.14 $\pm$ 0.22	6	—	—

Glucose uptakes expressed as increments over the basal glucose uptake $\pm$ S.E. of mean.

N is $N_1 + N_2 - 2$ where N_1 and N_2 are the number of hemidiaphragms used for determining the basal glucose uptake and the glucose uptake in the presence of plasma or other added material.

† All samples of plasma were random samples.

*Groen and Willebrands (personal communication) have more recently concluded that this variation in sensitivity is not seasonal.

in the plasma of severely diabetic patients and depancreatized dogs, by the rat-diaphragm technique, may be due to the presence of inhibitors in these plasma samples and not to their low insulin content. The fractionation and identification of the material in human plasma responsible for its insulin-like activity in the rat-diaphragm assay would appear to merit urgent attention.

Insulin Content of Normal Human Plasma*

The insulin activity of normal human plasma assayed by the rat-diaphragm technique reported in this paper was found to be 13 milliunits per ml. This is considerably greater than the value of 0.34 milliunit per ml. reported by Bornstein *et al.* (1951b), who used the A.D.H.A. rat assay for insulin. This discrepancy between animal methods and the rat-diaphragm technique described in this paper is illustrated by the A.D.H. rat assay (Table V). In this experiment normal human plasma, assayed by the rat-diaphragm method to contain 13 milliunits of insulin per ml., was found to have no hypoglycaemic effect when injected into A.D.H. rats, though 2 milliunits of insulin produced a highly significant fall of blood sugar when injected into the same animals under similar experimental conditions. This discrepancy could be due to insulin antagonists in human plasma which antagonize the hypoglycaemic action of insulin in animals but which do not antagonize the action of insulin upon the isolated rat diaphragm *in vitro*, or it could be due to non-specificity of the rat-diaphragm assay for insulin in plasma. Some evidence for the former belief is provided by the report of Weitz and Hagedorn (Hagedorn, 1952). These authors, utilizing the fall in blood sugar of alloxan-diabetic hypophysectomized adrenalectomized rats to assay insulin, reported the insulin activity of normal human plasma after acid alcohol extraction to be ten times the value obtained by Bornstein *et al.* (1951b) for untreated plasma. The high value could result from a separation of insulin from insulin antagonists in the plasma by the extraction procedure which these authors used.

The reason for the discrepancy between the values obtained for the insulin activity of normal human plasma by various workers who have used the rat-diaphragm assay for plasma insulin is not clear. It may be due to differences in specificity between diaphragms obtained from different strains of rat, since some workers have reported wide differences in the sensitivity of the isolated rat diaphragm to insulin. The high values reported in this paper are not due to minor modifications of the technique (Randle, unpublished experiments). Since Ottaway (1953) has reported that small quantities of growth hormone *in vitro* will enhance the glucose utilization of rat diaphragm, the high values for the insulin activity of normal human plasma reported in this paper may result from the presence of growth hormone in the plasma. The insulin activity of acromegalic plasma assayed by the rat-diaphragm method is considerably greater than the insulin activity of normal human plasma, and it may be that this difference is also attributable to the presence of growth hormone in the plasma (Randle, 1954a). The nature of the material responsible for the insulin-like activity of human plasma in the rat-diaphragm assay is being further investigated.

Summary

The claim of Groen *et al.* that normal human plasma will increase the glucose utilization of the isolated rat diaphragm has been confirmed and the evidence for attributing this effect to insulin in the plasma critically re-examined.

The rat-diaphragm technique has been critically explored as a quantitative assay for small amounts of

insulin and applied to the determination of the insulin activity of normal human plasma. By this technique the insulin activity of plasma collected from normal human subjects after a glucose meal was found to be approximately 13 milliunits per ml. This estimate of the insulin activity of normal human plasma is considerably greater than those previously reported by other workers. The possible reasons for this discrepancy are discussed in brief.

The importance of specificity in bioassays for plasma insulin is stressed.

The author wishes to express his gratitude to Professor F. G. Young for much helpful advice and criticism during the course of this work and the preparation of this paper; to Dr. N. R. Lawrie, of the John Bonnett Clinical Laboratories, Addenbrooke's Hospital, Cambridge, for the maintenance and sterilization of syringes; to Mr. G. Baker and Miss D. Cope for technical assistance in these experiments and for the preparation of the figures in this paper. It is a pleasure to thank Mr. D. R. Cox, of the Department of Mathematics, University of Cambridge, for assistance in the statistical analysis of the regression and assay data. The author is indebted to Boots Pure Drug Co., Ltd., for generous supplies of crystalline insulin.

The cost of this work was largely defrayed by an expenses grant from the Medical Research Council.

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Meat hygiene was the subject of a recent seminar in Copenhagen organized by W.H.O.'s regional office for Europe. The following figures are given for the consumption of meat (carcass weight) in a year per head of population in different countries. The figures relate to 1952-3 unless otherwise stated.

Country	In Kilograms	In Pounds
Austria	41	90
Belgium and Luxembourg	45	99
Canada (1951-2)	66	146
Denmark	57	126
Finland	32	71
France	60	132
Germany (Federal Republic)	39	86
Greece	13	29
Ireland (Republic)	53	117
Italy	16	35
Netherlands	32	71
Norway	33	73
Sweden	50	110
Switzerland (1951-2)	47	104
Turkey (1951-2)	16	35
United Kingdom	47	104
United States of America (1951-2)	76	168
Yugoslavia	18	40

Meat is easily contaminated, and is an important source of food poisoning. According to figures collected at the seminar, in the United States from 1945 to 1947 32% of the food-poisoning outbreaks were due to meat. In England and Wales in 1950, 68% of the outbreaks affecting more than one person in which the source was identified were due to contaminated meat.

*Bioassay methods for insulin in plasma almost certainly measure the resultant of the contra-actions of insulin and insulin antagonists present in the plasma. For this reason the term *insulin activity* has been used in this paper in referring to the biologically determined *insulin content* of plasma.