Determination of Serum Insulin Using the Isolated Rat Diaphragm

The Effect of Serum Dilution

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INTRODUCTION

The method for the determination of serum insulin, based on the stimulation of glucose uptake of the isolated rat diaphragm muscle by small quantities of insulin, has been used by several authors.[1-4] Although the method is relatively easy and reproducible, serious difficulties have been encountered in the effort to make it sufficiently accurate in studies of metabolic problems.

Accuracy is limited by (1) large variations in response of the tissue of different rats to the same dose of insulin and (2) the logarithmic relation between the effect on glucose uptake and the concentration of insulin. From statistical data it has been calculated by Randle[3] that the confidence limits of the determination are about three and one-third times the values found; these figures are in good agreement with our findings recorded below.

Another fact limiting accuracy is that the values reported by different authors for the insulin activity of normal serum vary widely. Vallance-Owen and associates[4] found less than 0.1 milliunits of insulin per ml. of plasma in the fasting state. Randle[3] reported values ranging from 10 to 20 milliunits per ml. in the plasma of healthy individuals, taken two and a half hours after 50 gm. of glucose by mouth. We have reported[2] figures for serum insulin of 0.1 to 3.0 milliunits per ml. while fasting and resting. These differences are far outside the confidence limits mentioned above and hence must be explained in some other way. Differences between plasma and serum, or between fasting and nonfasting subjects, do not seem to be relevant in this respect,[2] so that differences in technic seem to be the most likely explanation at the moment.

Although the technics used by the three above-mentioned authors are basically the same, there are minor differences. One of these is the dilution of serum during the incubation with the diaphragm. Groen and associates[1] and Randle[3] used serum diluted five to ten times with buffer; Vallance-Owen[4] used undiluted serum. It is possible that this variable is responsible for these discrepancies. Therefore, we made systematic investigations of this factor which we report in this paper.

METHODOLOGY

For these experiments the "pooled diaphragm technic" previously described by us[2] was rejected for the following reasons: (1) The effects obtained with concentrations of insulin of 0.1 to 1 milliunits per ml. of incubation medium were sometimes low, possibly because of the damage done to the tissue by dividing the muscle in four or five pieces and by the long time (fifteen minutes) required to collect the tissue from eight rats; (2) the theoretical advantage of pooling diaphragms of several rats often proved to be small in practice since standard deviations remained high; (3) last, but not least, the method required a great number of rats.

The following technic, using single hemidiaphragms, overcomes these difficulties to a certain extent and proved to be reliable within certain limits. It deviates from the old technics as follows:

1. Rats weighing 110±5 gm. were used. Liébecq[5] found that rats of this size show the greatest insulin effect when it is expressed as percentage increase of glucose uptake.

2. The whole diaphragm was carefully and quickly dissected, spread out on a piece of filter paper, blotted slightly to remove fluid, and divided with scissors into two approximately equal pieces.

3. The two hemidiaphragms were incubated, as described previously, in separate flasks containing 1 ml. of

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gassed (93 per cent O_2 + 7 per cent CO_2) buffer solution of 150 mg. per cent glucose. Depending on the object of the experiment both flasks either contained known but different concentrations of insulin, or one flask a known and the other an unknown amount of the hormone.

4. After ninety minutes of incubation, the glucose concentration in the medium was determined by the method of Hagedorn and co-workers.[6]

5. The effect of insulin was expressed as the increase of glucose consumption per hemidiaphragm instead of per 100 mg. wet tissue as described previously.

According to Liébecq[5] the glucose consumption of hemidiaphragms, with or without insulin, is quite independent of the weight of the diaphragms, thin hemidiaphragms of small rats taking up as much glucose as thick hemidiaphragms of heavy rats. Therefore, glucose uptake per gram of incubated tissue, times the weight of the tissue per flask, i.e., the glucose utilization per flask, is essentially constant. We have found that the values for glucose uptake per hemidiaphragm give more consistent results and smaller standard deviations than those calculated on the basis of tissue weight.

Experiments with different known concentrations of insulin showed: (1) A considerable variation in glucose uptake per hemidiaphragm of different rats with the same amount of insulin, as was anticipated; (2) a considerable day-to-day variation in the mean value of the glucose uptake of a group of rats receiving the same dose of insulin; (3) an approximate linear relationship between the logarithm of the insulin concentration and glucose uptake in the range of 0.03 to 8 milliunits of insulin per ml. of incubation medium (see table 1 and figure 1).

On the basis of these findings, the actual test is carried out in a number of parallel experiments by estimating the difference in glucose utilization of the two halves of the same diaphragm. One half is incubated with a known concentration (I_s), the other with the unknown concentration (I_x) of insulin. I_s is always taken in the middle of the linear range.

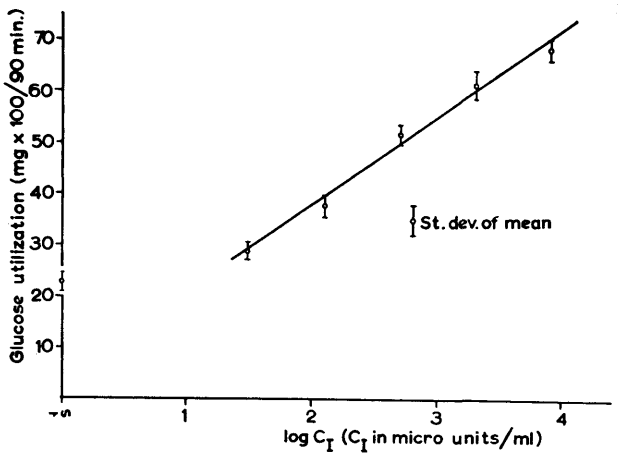

FIG. 1. Standard curve showing approximate linear relationship between glucose utilization (in mg. per half-diaphragm in 90 minutes) times 100 and the logarithm of the insulin concentration (in microunits/ml. incubation medium; 1 microunit = 10^{-6} unit).

Assuming the above-mentioned linear relationship between glucose uptake and the logarithm of the insulin concentration, and assuming also that the slope of this linear regression line is the same for all rats and for all days, the mean value of I_x can be calculated by the equation:

$$D_M = \frac{(G_s - G_x)_M}{b} \qquad (1)$$

in which D_M is the mean value of $\log \frac{I_s}{I_x}$

G_s = glucose uptake in medium with I_s

G_x = glucose uptake in medium with I_x

$(G_s - G_x)_M$ = mean value of the difference in glucose uptake in n tests with I_s and I_x

TABLE 1

Mean glucose utilization (mg. glucose x 10^2/90 min.) of hemidiaphragms incubated with different concentrations of insulin (mU/ml. incubation medium)

	Insulin concentration in mU/ml. incubation medium											
Date	0	n	0.03	n	0.125	n	0.5	n	2	n	8	n
6-12-55	14.6	5	20.0	5	27.6	5	48.0	5	61.1	5	67.8	5
12-12-55	34.6	5	35.4	5	41.2	5	56.0	5	71.4	5	65.4	5
4- 1-56	23.0	5	33.4	5	43.4	5	56.0	5	60.0	5	73.0	5
17- 1-56	19.0	5	27.0	5	38.4	5	48.2	5	51.6	5	66.2	5
over-all mean	22.8	20	28.9	20	37.7	20	51.7	20	61.2	20	68.1	20
SEM of over-all mean	2.0		1.7		2.1		1.9		2.7		2.1	

n=number of replicates; $SEM = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$

b = slope of regression line (v. figure 1).
The value of b can be obtained by performing the test with two rat diaphragm halves. These are incubated with known but different concentrations of insulin (I_{s1} and I_{s2}) at a fixed ratio, e.g., 4.

$$\text{In this case:} \quad \log \frac{I_{s1}}{I_{s2}} = 0.6$$

$$\text{Accordingly:} \quad b = \frac{(G_{s1}-G_{s2})}{0.6} \qquad (2)$$

The test is repeated over a range of insulin concentrations at the same ratio, i.e., 4. The mean value of b is then calculated by Equation 2.

For any estimated value of D_n of an unknown insulin concentration the confidence limits (D_1 and D_2) can be calculated by the formula:

$$D_{1 \text{ and } 2} = \frac{(G_s-G_x)_m}{b} \pm \frac{t}{b}\sqrt{(a)} \qquad (3)$$

in which:

$$(a) = \frac{(k-1)\,(sd\Delta G)_s^2+(n-1)\,(sd\Delta G)_x^2}{(k+n-2)\cdot n} \qquad (4)$$

where: t=Student's t which at F=k+n—2 degrees of freedom has a probability P.
k=number of replicates in test with I_{s1} and I_{s2}.
n=number of replicates in test with I_s and I_x.
$(sd\Delta G)_s$ and $(sd\Delta G)_x$ are standard deviations in k tests with $\frac{I_{s1}}{I_{s2}}$ and n tests with $\frac{I_s}{I_x}$ respectively.

The confidence limits for I_x are therefore

$$T_s \cdot 10^{D_1} \quad \text{and} \quad T_s \cdot 10^{D_2}*$$

The slope b has been determined on several occasions in the course of one year, using the above-mentioned known doses of insulin with a ratio of 4 (D=0.6). While the values of b found in the range 0.125—2 milliunits per ml. were rather constant and in rather good agreement with that obtained from figure 1, those obtained in later times on the lower and higher ends of the regression line were often considerably smaller, indicating a possible curvature on both extremes. We adhered to the described method, however, since the range 0.125—2 was the one most commonly used and the standard used was always 0.5 milliunits/ml. The value for b used was calculated from all the figures of $(G_{s1}-G_{s2})$ obtained in that same range up till the day of the test in question. After some time b tended to become constant at a value of about 18.

Editorial Note: An alternative method for the calculation of the confidence limits of I_x using terminology more familiar to American readers is as follows:

Let R_m=mean value of n tests of $\frac{(I_s)}{(I_x)_m}$

Then the confidence limits $R_{1 \text{ and } 2}$ for given value of Student's t are expressed by the equation:

$$\log R_{1 \text{ and } 2} = \log R_M \pm t \cdot (SEM)_R \qquad (5)$$

where: $(SEM)_R$=standard error of the mean value of log R_m.
But the pooled variance for the calculation of $(SEM)_R$ for k determinations of $\Delta G_s=(G_{s1}-G_{s2})$ and n determinations of $\Delta G_x=(G_s-G_x)$ is:

$$(sd\Delta G)^2 \text{ pooled} = \frac{(k-1)\,(sd\Delta G)_s^2+(n-1)\,(sd\Delta G)_x^2}{(k+n-2)}$$

$$\text{Accordingly} \quad (SEM)_R = \frac{1}{b}\sqrt{\frac{(sd\Delta G)^2_{\text{pooled}}}{n}}$$

From Equation (5) the ratio of the confidence limits to R_{mean} is:

$$\frac{R_{1 \text{ and } 2}}{R_{mean}} = \frac{(I_x)}{(I_x)_{mean}} = 10^{\pm t \,\cdot\, (SEM)_R}$$

In the example given in the paper t=2 and $(SEM)_R$ =0.25; therefore the ratios of the confidence limits $I_{1 \text{ and } 2}$ to I_m are:

$$\frac{I_{1 \text{ and } 2}}{I_m} = 10^{\pm 0.5} = 3 \text{ and } 1/3$$

(For a discussion of the use of pooled variance in determining confidence limits of a value calculated from two groups of measurements see: Snedecor, George, *Statistical Methods*, chapter 4, Iowa State College Press, Ames, Iowa, 1946.)

Standard deviation $(sd\Delta G)_s$ was rather high: In April 1956 it was 10.6 (k=47); in September 1956 it was 9.6 (k=90) and changed very little afterwards. In nearly every test five animals were used (n=5) and therefore the confidence limits became practically constant, (a) being almost completely determined by k and $(sd\Delta G)_s$. With k=90, n=5, $(sd\Delta G)_s$=10 and $(sd\Delta G)_x$ = about 7, (a) becomes about 20. Taking P=0.05, t is 2.0 (F=93) and therefore $t/b\sqrt{a}$ becomes about 0.5 and the confidence limits $10^{0.5}=3$ and $10^{-0.5}=1/3$. This means that when a certain value I is

*estimates of variance used are

$$SD = \text{standard deviation} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{(n-1)}}$$

$$SEM = \text{standard error of mean} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$$

found for an unknown concentration, the probability is 95 per cent that the true value is between 1/3 I and 3 I.

For the determination of the insulin activity of serum it was diluted to the desired extent by adding calculated amounts of buffer without glucose and/or buffer containing 500 mg. per cent of glucose in such a way that the glucose concentration in the buffer-serum mixture is 150 mg. per cent. The insulin activity of this mixture is tested as described above, using one hemidiaphragm of a rat, and a standard insulin solution in buffer (usually 0.5 mU/ml.) is tested using the other hemidiaphragm. For every determination at least five animals are used. The calculation of the insulin activity in the serum-buffer mixture was carried out as described above. From this the serum insulin concentration per ml. is found by multiplication by the dilution-factor.

The sera used were obtained from normal individuals and patients not suffering from diseases that interfere with carbohydrate metabolism. Blood was taken early in the morning while fasting. Dilutions more than fifty times have not been used because the effect to be measured became too small.

RESULTS

In table 2 and figure 2 the results of these experiments are given. Insulin concentrations found are indicated on the ordinate on a logarithmical scale with their confidence limits for P=0.05.

In all cases the serum insulin values found with high percentages (100 to 50 per cent) of serum are considerably less than those found with smaller percentages (5 to 10 per cent). In most cases the differences between the values obtained with the highest and the smallest serum concentration used were statistically significant, as there was no overlapping of the ranges of probability. In three of the six cases the serum insulin values obtained with nearly undiluted serum were close to those given by Vallance-Owen et al.[4] and all of them were below 1 milliunit/ml.

It seems justified to conclude from these data that the low values for normal serum insulin, recorded by the last-mentioned authors, are at least in part due to the fact that they use undiluted serum for the determination.

As to an explanation for this "dilution phenomenon" at least two possibilities have to be considered. In the first place one could suggest that undiluted serum is an unfavorable, unphysiological medium for the diaphragm tissue and that the high protein concentration causes a diminished ability of the muscle cells to take up glucose. A second explanation would be that the serum contains

TABLE 2

Case	Age	Diagnosis	Per cent serum in incub. med.	Insulin activity (mU/ml.)	Confidence limits (P=0.05)
C	Male	72 Hypertension	50	0.3	0.1 – 0.8
			20	1.2	0.5 – 2.9
			10	1.6	0.7 – 4.1
Z	Male	60 Hypertension	50	0.7	0.3 – 1.5
			20	0.9	0.4 – 2.0
			10	4.0	1.8 – 9.0
Ha	Male	48 Ulcus jejuni	70	0.9	0.4 – 2.1
			20	2.5	1.1 – 6.0
			10	4.6	2.0 –10.8
			5	3.1	1.4 – 7.3
			2	3.4	1.5 – 7.9
Ho	Male	64 M. Bechterew	90	0.1	0.04– 0.3
			10	1.3	0.5 – 3.6
			2	2.3	0.8 – 6.4
R	Male	27 Empyema pleurae	50	0.4	0.15– 1.1
			10	1.4	0.5 – 3.9
			2	3.4	1.2 – 9.5
Ru	Male	33 normal	85	0.1	0.03– 0.3
			10	0.9	0.3 – 2.7
			5	1.1	0.35– 3.3
			2	0.4	0.1 – 1.2

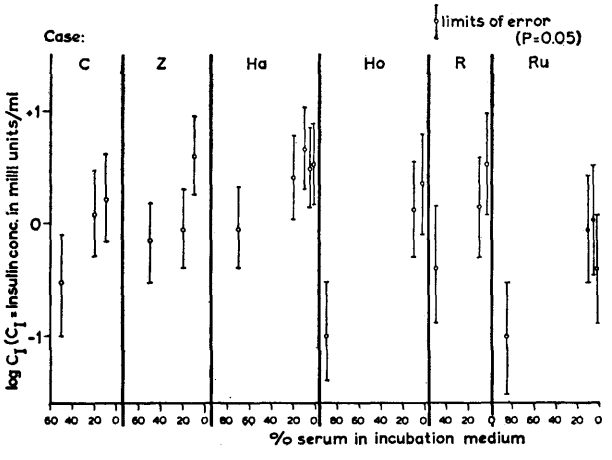

FIG. 2. This figure shows variation in serum insulin values found with different dilutions of serum. On the horizontal: percentage of serum in the medium used for the incubation of the diaphragm tissue; on the ordinate (on a logarithmical scale): serum insulin values found in milliunits per ml. of serum with confidence limits for P = 0.05.

substances counteracting the effect of the serum insulin, and that the activity of these inhibiting substances diminishes more rapidly with dilution than the effect of the serum insulin.

We have found no evidence in favor of the first assumption. On the contrary, we have observed that the effect of pure insulin is not diminished by addition of relatively high concentrations of pure serum albumin.

TABLE 3

Influence of serum albumin* on effect of insulin in the rat diaphragm test

Medium	Albumin %	Insulin mU/ml.	Glucose ut. mg. x 10^2 90 min.	n	Effect of albumin	SEM (1)	P	
1) Buffer	–	–	25.8	8	–	–		–
2) Buffer	3.5	–	34.4	8	+8.6	2.1	P 1.2	<0.01 >0.001
3) Buffer	–	0.5	56.8	9	–	–		–
4) Buffer	3.5	0.5	62.0	9	+5.2	4.7	P 3.4	>0.10

* 1.25 gm. of serum albumin (Fraction V acc. Cohn) dissolved in 25 ml. of oxygen-free buffer (Gey and Gey) *p*H 7, containing 100 mg. per cent glucose, was dialyzed in Visking cellophane first for two days with agitation at 0° C. against 21 of the same buffer containing in addition 250 mg. per cent of cystein, and afterwards for another two days under the same conditions against the same buffer solution but without cystein and with 150 mg. per cent of glucose. The final solution (±35 ml.) containing about 3.5 per cent of albumin was used for the experiment.

(1) $SEM=\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$

In table 3 the results of some experiments of this kind are recorded. A solution of 5 per cent human serum albumin (fraction V of Cohn) was purified by dialysis against buffer containing cystein, to inactivate possible traces of insulin, and the solution obtained, containing about 3.5 per cent of protein, was tested against buffer with and without added insulin (0.5 milliunits/ml.). The albumin itself showed a slight positive effect, comparable with about 0.05 milliunit per ml. of insulin, but it had no significant influence on the effect of added insulin.

As to the second possible explanation for the "dilution phenomenon" we have found that the insulin activity of serum is suppressed by the hormones of the adrenal medulla even in very low concentrations and that the degree of this inhibition is indeed dependent on the dilution of the serum. Experiments dealing with this problem will be published in another publication.[7]

DISCUSSION

It is evident that the elucidation of this problem is of more than methodological importance, as it is directly significant for the problem of the level of normal serum insulin. We are inclined to accept the values obtained after dilution as the more likely ones since figure 2 shows that the values obtained tend to become constant at higher dilutions.

The degree of dilution that was originally described by us (1): five to ten times, and which was later also used by Randle, should therefore be standardized to a dilution of ten times, so that the results of different investigators might better be compared.

SUMMARY

A new modification of the rat diaphragm method for the determination of serum insulin is described in which hemidiaphragms of five rats weighing 110±5 gm. are used.

The values found for serum insulin by this method depend on the concentration of serum in the medium during the determination, lower concentrations yielding higher insulin values.

Possible explanations for this phenomenon are discussed.

This finding explains some of the differences in the values for normal serum insulin as found by different authors.

SUMMARIO IN INTERLINGUA

Le Influentia Del Dilution Del Sero Super Le Determination Del Concentration De Insulina In Le Sero Per Medio De Isolate Diaphragmas De Ratto

Es describite un nove modification del utilisation de diaphragmas de ratto in le determination del concentration de insulina in le sero. Le innovation es le uso de hemidiaphragmas ab cinque rattos que pesa 110±5 g.

Le valores pro insulina seral trovate per iste methodo depende del concentration de sero in le medio durante le processo del determination. Plus basse concentrationes de sero resulta in plus alte valores de insulina. Le explicationes possibile de iste phenomena es discutite. Le phenomeno mesme explica certes del differentias inter le valores pro normal concentrationes de insulina in le sero que ha essite reportate per differente autores.

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Fat Absorption in Man

Opportunities for studying fat absorption in man are uncommon since they depend upon an opportunity to examine thoracic duct chyle. M. J. Albrink, W. W. L. Glenn, J. P. Peters, and E. B. Man (*J. Clin. Invest.* *34*:1467 (1955)) were able to perform detailed analyses of the lipids of chyle in five patients with thoracic duct fistulas. The emulsified and soluble lipids were analyzed separately, the division being achieved by slow centrifugation. The concentrations of the lipid components of the creamy layer were calculated as the differences of the values obtained from analyses of the lipid concentrations of the whole fluid and the subnatant following centrifugation.

The lipids of chyle were largely present in the emulsified state. Neutral fat comprised the bulk of the particulate matter, but a small, fairly constant concentration of neutral fat of 6.0 to 10.8 milliequivalents per liter was found in the subnatant. Cholesterol and phospholipids were divided between both fractions, and the ratio of free cholesterol to cholesterol ester was similar in both layers.

One patient was studied after periods of fat feeding and fat restriction. Neutral fat in the emulsified layer increased and decreased with the fat content of the diet. This is in agreement with an earlier study of J. P. Peters and E. B. Man (*Metabolism* 2:30 (1953)) in which it was shown that the lipid pattern of chyle from a patient maintained on intravenous feedings resembled that of serum.

Analyses of the creamy layer disclosed little protein; the amount found was within the error of the method of analysis. Thus, the lipids in this layer are probably in their native state. The cholesterol and phospholipids may be assumed to be dissolved in the neutral fat. In the clear subnatant layer the lipids are held in solution by lipoproteins, most probably synthesized by the liver. When fat is withheld from the diet the chyle and serum lipids are alike, due to the hepatic release of lipoproteins.

In the postabsorptive state the subnatant lipids of chyle vary markedly from those of serum. The cholesterol concentration is lower and neutral fat is higher than in plasma. There is considerable evidence suggesting that a cholesterol-phospholipid-protein complex acts to convey lipids to tissue. The lipids of the subnatant fluid thus reflect the lipoprotein complexes as they are formed and the large difference in neutral fat concentration may be attributed to rapid utilization by the peripheral tissues. These findings in man have essentially confirmed the earlier similar hypotheses based upon experimental studies with animals.

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