IMMUNOLOGICAL STUDIES WITH CIRCULATING INSULIN

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While the chemical structure of insulin as extracted from the pancreas is now known (Sanger, 1960), our knowledge of the form in which insulin circulates in the blood-stream remains fragmentary. It would be surprising if the changes of physico-chemical environment as a result of passing through the liver, lungs, and endothelial lining of the capillaries did not in some way modify the properties and also the biological effects of insulin. Knowledge of the possible modifications of insulin after it leaves the pancreas is clearly important in our understanding of conditions such as diabetes mellitus. In this communication we describe experiments which suggest that there is an immunological difference between circulating and extracted pancreatic insulin.

Methods and Material

Assay Procedure (Modified from Martin, Renold, and Dagenais, 1958)

Fed male Wistar rats weighing 125–130 g. were killed by cervical fracture and then bled by partial decapitation. The epididymal fat tissue was floated out in Krebs-bicarbonate buffer containing D-glucose (300 mg./100 ml.), at room temperature. The leafy portion of each fat pad was cut longitudinally into six or seven pieces weighing 10–20 mg. each. These were visually grouped for comparability of surface area, pieces from different groups then being used to make up the set of tubes for any particular estimation.

The pieces of fat tissue were placed in 1 ml. of incubating medium, contained in wide-necked glass tubes carrying a side-arm (15 ml. in volume). A large rubber stopper with a central diaphragm closed the mouth of the tube and an all-glass cup of 1.5-ml. capacity was suspended from the under-surface. Smaller gas-tight rubber bungs were inserted into the side-arm.

The system was equilibrated with 5% CO₂ and 95% O₂ at 37° C. for 5–10 minutes and was then sealed off, all subsequent manipulations being made via needles attached to spring-loaded syringes. Finally, 1 ml. of gassed Krebs-bicarbonate buffer at 37° C. containing 0.2 µC ¹⁴C-1-glucose was introduced through the side-arm, making a total volume of 2 ml. of incubating medium.

The Krebs buffer or other incubating media were invariably made up to a final glucose concentration of 300 mg./100 ml. and gelatin (200 mg./100 ml.) was added.

The incubation was carried out in a Dubnoff metabolic tank at 37° C. for 120 minutes at 70–80 oscillations per minute. Then 1 ml. of a 1 M solution of hydroxide of "hyamine" (*p*-(diisobutyl-cresoxythoxyethyl)dimethyl benzyl ammonium hydroxide) in

methanol was placed in the central hanging cup and 0.2 ml. of 1 N H₂SO₄ introduced into the incubating medium. After cooling the bath water to approximately 25° C. the tubes were shaken at 120–130 oscillations per minute for three hours. The hanging cups were then detached, and, after washing the outside thoroughly in water, placed directly into 10 ml. of toluene scintillator (2,5-diphenyloxazole (POP) 4 g. and 1,4-bis-(2-(5-phenyloxazolyl))-benzene (POPOP) 50 mg./l.) contained in low-background glass counting-phials. The hyamine was mixed carefully with the scintillation fluid and the cup removed.

The fat tissue was washed thoroughly, blotted, and dried in a desiccator overnight in pre-weighed tubes. The tissue was then weighed and homogenized into 20 ml. of ethanol:petroleum ether, 3:1. After centrifuging, the supernatant was well shaken with 10 ml. of normal saline and allowed to separate at 4° C. 3-ml. aliquots of the upper layer were placed in pre-weighed counting-phials and the petroleum ether was evaporated with a jet of nitrogen. After removing traces of water by drying overnight in a desiccator, the fat in the phials was weighed and 10 ml. of the liquid scintillator added.

The samples were counted in a Packard "tri-carb" liquid scintillation spectrometer at high-voltage tap 10 (1,365 volts) with lower discriminator bias settings of 10 volts. The proportion of the total counts recorded between 10 and 60 volts serves as a monitor for excessive quenching. The results are expressed as counts per minute per $\sqrt{\text{dry weight of tissue}}$ (for CO₂) or fat per 120 minutes (c/m/ $\sqrt{\text{dw}}$ /120 min.).

Preparation of Insulin Extracts.—Extracts of serum and of pancreatic tissue were made according to the method of Jephcott (1931) as modified by Grodsky and Forsham (1960).

Preparation of Insulin Antibody.—Immune guinea-pig serum against $\times 6$ recrystallized insulin (Boots Pure Drug Co. Ltd.) was prepared according to the method of Moloney and Coval (1955) as modified by Wright (1959). In all experiments in which the effect of insulin antibody was assessed control guinea-pig serum was obtained either from normal animals or from animals injected with the inoculum alone (lanolin and Freund's adjuvant). The potency of these antisera naturally varied, but a considerable excess of antibody was always used. All the immune guinea-pig antisera employed in these experiments were shown to antagonize fully the effect of 900–1,000 µU/ml. of crystalline ox insulin at, or at less than, the concentrations used in the serum or serum extract experiments. Most of the immune serum samples had also been shown to produce transient hyperglycaemia when injected into rats and to be capable of agglutinating insulin-coated red blood cells (Robinson and Wright, 1961).

Results

Fat-pad Insulin-assay Procedure's Sensitivity and Precision.—The method appears to be highly sensitive to pancreatic insulin. The Table shows the values obtained for the basal tissue activity (without insulin) and its response to a standard amount of crystalline ox insulin (900–1,000 µU/ml.—that is, 1,800–2,000 µU/tube). This standard insulin dose increases the rate of ¹⁴CO₂ production by tenfold to elevenfold, and the rate of ¹⁴C-fat formation (ether-extractable material) by twelvefold to thirteenfold. A smaller number of

observations were made with a very low insulin concentration (8 μ U/ml. or 16 μ U/tube). These showed a mean rise of 90% over the basal values ($P < 0.001$).

Further studies of the dose response relationship of the method are in progress, but the figures reported above suffice for the interpretation of the present experiments.

The measurements are also reasonably precise. For the rate of $^{14}\text{CO}_2$ production the coefficient of variation of the 39 basal observations was 31%, and of the 37 observations with the standard insulin concentration 30%. Corresponding values for the rate of ^{14}C -fat formation were 39% basally and 35% with insulin. These figures refer to the results obtained in all experiments, although within any one experiment (when only a single animal is used—12–15 tubes) the variability is much less. It will be noted that the variability obtained with rat epididymal fat tissues by our technique compares favourably with rat-diaphragm method of insulin assay (Groen *et al.*, 1952; Randle, 1954; Vallance-Owen *et al.*, 1955) despite the greater sensitivity.

Effect of Guinea-pig Antiserum on Insulin-like Activity of Crude and Crystalline Extracted Pancreatic Insulin.—First a study was made of the effect on the subsequent fat-pad assay of adding immune guinea-pig serum to the crystalline ox insulin or to other crude insulin preparations obtained by making acid-ethanol extracts of human, ox, and dog pancreas. In all instances the stimulating effect of these various pancreatic insulins on both ^{14}C -fat formation and on $^{14}\text{CO}_2$ production was abolished completely by the antiserum when compared with the values obtained after adding control or non-immune guinea-pig sera. Figs. 1 and 2 illustrate this inhibitory effect of insulin antibody on the rate of ^{14}C -fat and $^{14}\text{CO}_2$ formation when incubations were made with crystalline ox insulin (Fig. 1) or with crude human or ox insulin (Fig. 2). Preliminary results indicate that this is also true of crude guinea-pig insulin; yet, in accordance with the work of others (Moloney and Coval, 1955; Wright, 1959), none of the immunized guinea-pigs became diabetic.

Effect of Guinea-pig Antiserum on Insulin-like Activity of Whole Serum.—In contrast to the effect on pancreatic insulin preparations, the insulin-like activity found in samples of whole serum obtained postprandially from either normal humans or the cow was found to remain unimpaired when incubations were carried out with immune guinea-pig serum. This was true for both the rate of ^{14}C -fat formation and $^{14}\text{CO}_2$ production. In the case of the ox serum experiments a relatively high concentration of guinea-pig antiserum was used ($\frac{1}{4}$), and any question of species difference was obviated because ox insulin had been injected to immunize the guinea-pigs.

Effect of Insulin on Rate of ^{14}C -fat Formation and $^{14}\text{CO}_2$ Production from ^{14}C -1-Glucose by Rat Epididymal Fat Tissue

	^{14}C Fat Formation†				$^{14}\text{CO}_2$ Production†			
	No. of Obs.	Mean	Coef. Var.*	S.E. of Mean	No. of Obs.	Mean	Coef. Var.*	S.E. of Mean
Basal tissue activity	34	99	39%	6.6%	39	126	31%	4.8%
Insulin 8 μ U/ml. buffer	8	187	13%	4.5%	—	—	—	—
Insulin 990–1,000 μ U/ml. buffer	31	1,277	35%	6.3%	37	1,344	30%	5.0%

* Coefficient of variation.

† Activity expressed as counts per minute per dry weight fat (^{14}C -fat formation) or tissue ($^{14}\text{CO}_2$ -production) per 120 minutes.

Effect of Guinea-pig Antiserum on Activity of Serum Extracts.—In order to increase the specificity of the method for the detection of insulin in serum, and in order to reduce the effect of possible substances in the serum which could interfere with the antigen-antibody reaction, postprandial sera from man and from the cow were extracted by the same method employed for the extraction of insulin from the pancreas. When these were incubated with and without antibody, there was again no demonstrable effect of the immune guinea-pig serum on the rate of ^{14}C -fat formation (Fig. 3). $^{14}\text{CO}_2$ production was also unaffected.

Effect of Guinea-pig Antiserum on Activity of Serum or Serum Extracts with Added Crystalline Ox Insulin.—To assess further whether there were substances in serum which could interfere with the antibody-antigen reaction, crystalline ox insulin was added to whole or extracted serum and the biological effect measured with and without anti-insulin serum. It was found that the insulin-like activity of whole human serum to which crystalline ox insulin had been added *in vitro* 500 μ U/ml.) could be reduced by immune guinea-pig serum to the level produced by serum alone. Similar findings were obtained with serum taken from a newly discovered diabetic patient of the juvenile variety, four hours after a subcutaneous dose of soluble ox insulin—that is, with insulin added *in vivo*. Finally, crystalline ox insulin was added to serum extracts from blood collected from a dog four days after total pancreatectomy, when the

FIG. 1.—Effect of guinea-pig antiserum on the "fat-pad" activity of crystalline ox insulin.

FIG. 2.—Effect of guinea-pig antiserum on the "fat-pad" activity of acid-ethanol extracts of human and ox pancreas. Each dot represents a single estimation.

FIG. 3.—Effect of guinea-pig antiserum on the "fat-pad" activity of acid-ethanol extracts of postprandial human and ox serum. Each dot represents a single estimation.

FIG. 4.—Effect of guinea-pig antiserum on the "fat-pad" activity of acid-ethanol extracts of serum with added crystalline ox insulin. Blood was collected four days after total pancreatectomy in a dog. Results of two separate experiments.

FIG. 5.—Effect of total pancreatectomy on the ability of whole serum to stimulate ^{14}C -fat formation from ^{14}C -1-glucose.

blood-sugar concentration had risen to 997 mg./100 ml., and when the serum alone gave no detectable insulin-like activity as measured by ^{14}C -fat formation. Fig. 4 shows that when crystalline insulin was added in amounts likely to be present physiologically (250 $\mu\text{U}/\text{ml.}$), the insulin-like activity of the serum-insulin mixture could still be abolished by immune guinea-pig serum.

Effect of Total Pancreatectomy on Serum Insulin-like Activity.—Evidence which suggests that the capacity of whole serum to stimulate ^{14}C -fat formation by rat epididymal fat tissue from ^{14}C -1-glucose depends largely upon the presence of insulin was obtained by studying the effect of total pancreatectomy on the insulin-like activity in the dog's serum. Fig. 5 shows that by the fourth post-operative day (when the blood-sugar level had reached 997 mg./100 ml.) the insulin-like activity, as measured by ^{14}C -fat formation, had fallen progressively to within the range of the basal estimations. But, in accordance with the work of Leonards (1959), this was not true of $^{14}\text{CO}_2$ production. The mean fourth-day serum activity as measured by this means was 337 c/m/ $\sqrt{\text{dw}}$ tissue/120 min., nearly three times higher than the mean basal figure for these experiments (119 c/m/ $\sqrt{\text{dw}}$ tissue/120 min.).

Discussion

Winegrad and Renold (1958) have shown that pancreatic insulin *in vitro* will greatly increase the rate of both $^{14}\text{CO}_2$ production and ^{14}C -fat formation from ^{14}C -1-glucose by rat epididymal fat tissue. Our results confirm this and also the insulin sensitivity of the procedure, as Martin *et al.* (1958) have also found. However, whether the stimulating effect of serum or plasma on rat epididymal fat is due wholly, or at least largely, to the presence of circulating insulin is difficult to prove (Leonards, 1959).

Our results with antiserum to ox insulin prepared in the guinea-pig indicate that the insulin-like activity (as measured by both ^{14}C -fat formation and $^{14}\text{CO}_2$ production from ^{14}C -1-glucose) of either whole serum or acid-ethanol serum extracts is little, if at all, inhibited by the presence of an insulin antibody in the incubating medium. This result stands in striking contrast to the results reported by Taylor and Randle (1959) using rat hemidiaphragms. They found complete inhibition of serum-insulin-like activity (glucose uptake) when whole ox serum was incubated in the presence of a similarly prepared antiserum to ox insulin. Our results might suggest that the insulin-like activity of post-absorptive serum, whether assayed whole or in acid-ethanol extracts by the rat epididymal fat-assay method, is not due to the presence of circulating insulin; but this is unlikely.

Firstly, the method is highly sensitive to insulin, and all our sera were collected after a carbohydrate meal, when insulin would be expected in the circulating blood and can be detected by immunochemical assay (Grodsky and Forsham, 1960; Yalow and Berson, 1960). Even if relatively small amounts of insulin are present in serum the method should detect it in postprandial serum. Secondly, after total pancreatectomy the dog's serum-insulin-like activity as measured by ^{14}C -fat formation fell to within the range of the basal values; which is strong presumptive evidence that the internal secretion of the pancreas is being measured.

Although antagonism to the effect of insulin on rat-diaphragm muscle has been demonstrated after total pancreatectomy in the cat (Vallance-Owen and Lukens, 1957), this may not apply to adipose tissue. Following the methods of Vallance-Owen *et al.* (1958), C. Lowy, G. Blanchard, and D. Phear (1960, personal communication) have shown that albumin extracts of plasma proteins precipitated by trichloroacetic acid do not affect the action of insulin on rat epididymal fat tissue (glucose uptake and $^{14}\text{CO}_2$ production).

In confirmation of the work of Leonards (1959) after total pancreatectomy the rate of $^{14}\text{CO}_2$ production remains above the basal values, and it is for this reason that the data obtained in the present experiments have been illustrated by the effect on ^{14}C -fat formation. Further work is clearly needed to clarify the nature of this difference. High concentrations of adrenaline, cortisol, and growth hormone will stimulate $^{14}\text{CO}_2$ production from ^{14}C -1-glucose, although ^{14}C -fat-formation is either unaffected or depressed (Winegrad *et al.*, 1959; Lynn *et al.*, 1960; Hagen and Ball, 1960; Jeanrenaud and Renold, 1960).

Thirdly, further evidence that the effect of serum on rat epididymal fat tissue is at least largely due to insulin is provided by Owen (1960) and Renold *et al.* (1960), who find that reduced glutathione, which inactivates insulin, will also inactivate the effect of serum on the rate of $^{14}\text{CO}_2$ production by rat epididymal fat tissue. Finally, Renold *et al.* (1960) report a twofold to fourfold increase of serum insulin-like activity ($^{14}\text{CO}_2$ production from ^{14}C -1-glucose) after intravenous glucose administration.

Thus the activity of serum or serum acid-ethanol extracts on the rat epididymal fat pad is probably largely due to insulin but is little if at all antagonized by insulin antibody. It is possible that there are substances present in serum which interfere with the union of insulin and its antibody, but Moloney and Coval (1955), Arquilla and Stavitsky (1956), and Yalow and Berson (1960) have produced good evidence that at least injected insulin and some endogenous insulin will unite with antiserum to crystalline insulin prepared in the guinea-pig. Furthermore, the stimulating effect of ox serum on the *in vitro* glucose uptake by the rat diaphragm is completely inhibited by immune guinea-pig serum (Taylor and Randle, 1959). Thus lack of union between insulin and its antibody is unlikely as an explanation of our findings. Clearly, therefore, there is a difference, demonstrable immunologically, between insulin extractable from the pancreas and that extractable from the serum of the same animal. This conclusion receives confirmation from the work of Moloney and Coval (1955) and Moloney and Aprile (1959), who have shown that antiserum to crystalline ox insulin prepared in the sheep, rabbit, and horse will inactivate the biological effect (mouse-convulsion procedure) of insulin extracted from the pancreases of these same animals, none of which became diabetic. The nature and physiological significance of this immunological difference is at present being studied.

Summary

Rat epididymal fat tissue has been used *in vitro* for the assay of insulin and insulin-like activity by measuring the rate of production of $^{14}\text{CO}_2$ and ^{14}C -labelled fat from ^{14}C -1-glucose.

The stimulating effect of crude pancreatic extracts from various animals on both these parameters of

insulin action was completely abolished by an insulin antiserum.

Postprandial serum, whether whole or as an acid-ethanol extract, enhanced both $^{14}\text{CO}_2$ production and ^{14}C -fat formation, but this insulin-like activity was not affected by insulin antiserum.

After pancreatectomy in the dog, the stimulating effect of the whole serum on ^{14}C -fat formation was lost, suggesting that this effect of normal serum is due to insulin.

These findings suggest that there is a difference, demonstrable immunologically, between circulating and pancreatic insulin.

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REFERENCES

- Arquilla, E. R., and Stavitsky, A. B. (1956). *J. clin. Invest.*, **35**, 458, 467.
 Grodsky, G. M., and Forsham, P. H. (1960). *Ibid.*, **39**, 1070.
 Groen, J., Kamminga, C. E., Willebrands, A. F., and Blickman, J. R. (1952). *Ibid.*, **31**, 97.
 Hagen, J. H., and Ball, E. G. (1960). *J. biol. Chem.*, **235**, 1545.
 Jeanrenaud, B., and Renold, A. E. (1960). *Ibid.*, **235**, 2217.
 Jephcott, C. M. (1931). *Trans. roy. Soc. Canad.*, **25**, Sect. V, p. 183.
 Leonards, J. R. (1959). *Fed. Proc.*, **18**, 272.
 Lynn, W. S., MacLeod, R. M., and Brown, R. H. (1960). *J. biol. Chem.*, **235**, 1904.
 Martin, D. B., Renold, A. E., and Dagenais, Y. M. (1958). *Lancet*, **2**, 76.
 Moloney, P. J., and Aprile, M. A. (1959). *Canad. J. Biochem.*, **37**, 793.
 — and Coval, M. (1955). *Biochem. J.*, **59**, 179.
 Owen, J. (1960). *Diabetes*, **9**, 325.
 Randle, P. J. (1954). *Brit. med. J.*, **1**, 1237.
 Renold, A. E., Steinke, J., and Antoniadis, H. N. (1960). *Acta Endocr. (Kbh.)*, **35**, Suppl. 51, p. 1235.
 Robinson, B. H. B., and Wright, P. H. (1961). *J. Physiol. (Lond.)*, **155**, 302.
 Sanger, F. (1960). *Brit. med. Bull.*, **16**, 183.
 Taylor, K. W., and Randle, P. J. (1959). *J. Endocr.*, **19**, 221.
 Vallance-Owen, J., Dennes, E., and Campbell, P. N. (1958). *Lancet*, **2**, 696.
 — Hurlock, B., and Please, N. W. (1955). *Ibid.*, **2**, 583.
 — and Lukens, F. D. W. (1957). *Endocrinology*, **60**, 625.
 Winegrad, A. I., and Renold, A. E. (1958). *J. biol. Chem.*, **233**, 267, 275.
 — Shaw, W. N., Lukens, F. D. W., Stadie, W. C., and Renold, A. E. (1959). *Ibid.*, **234**, 1922.
 Wright, P. H. (1959). *Biochem. J.*, **71**, 633.
 Yalow, R. S., and Berson, S. A. (1960). *J. clin. Invest.*, **39**, 1157.

A manuscript item recently acquired for the History of Medicine Division of the National Library of Medicine is a ledger into which are pasted some sixty documents covering the service of Dr. Edward B. Dalton (1834–1872) with the U.S. Volunteers from the State of New York during the Civil War. A letter of special interest is dated from Georgetown in October, 1864; it is in an uneducated hand and apparently concerns one of Dr. Dalton's patients. Addressed "dear Friend Abreham Lincon," it reads: "as my husband is in your serviz and is sick and the docter ses that he never will bea no beter . . . if you please send him his discharge as he can do you three times as much good here in the comen election as his influence is greate here and i feal ancies to have him here as i dont want the trater mack [George Brinton McClellan] in the chare . . ." (*National Library of Medicine News*, May, 1961.)