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ORIGINALS

A Serum Inhibitor of Insulin Action on Muscle

I. Its Detection and Properties*

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Summary. 1. The intraperitoneal assay of insulin activity has been used to detect the presence of an insulin inhibitor in serum. 2. A solution of 5% bovine serum albumin has been established as an injection medium permitting the maximum insulin effectiveness. 3. When insulin was injected intraperitoneally in serum from male rats or male human subjects, the insulin effectiveness on the diaphragm was reduced compared with that of insulin in 5% bovine albumin; whereas that on the epididymal adipose tissue was unaffected. 4. The inhibitory activity was associated with the albumin fraction of the serum; and neither the inhibitory activity of the serum nor that of the albumin derived from the serum was affected by prolonged dialysis. 5. The inhibitory activity was released from the albumin by boiling at pH 4 to 5. The inhibitory substance appears to be a polypeptide of mol. wt between 5000 and 10000.

Un inhibiteur sérique de l'action de l'insuline sur le muscle: I. Sa détection et ses caractéristiques.

Résumé. 1. Le dosage biologique de l'activité insulinique selon la technique de l'injection intrapéritonéale a été utilisé pour détecter l'existence d'un inhibiteur sérique de l'insuline. 2. Il a été établi que la solution d'injection permettant d'obtenir un effet insulinique maximum est celle qui contient de l'albumine bovine, à raison de 5 g pour cent. 3. On constate que l'insuline mélangée, soit à du sérum humain normal, soit à du sérum de rats mâles normaux, est moins efficace dans son action sur le diaphragme que l'insuline injectée seule dans la solution d'albumine bovine à 5%. L'effet sur le tissu adipeux par contre demeure inchangé. 4. L'activité inhibitrice ainsi observée est associée à la fraction albumine du sérum. Elle ne disparaît ni après dialyse prolongée du sérum complet ni après celle de la fraction albumine seule. 5. L'activité inhibitrice est libérée de l'albumine quand on fait bouillir celle-ci, le pH étant de 4 à 5. Il semble que la substance obtenue de cette manière soit un polypeptide d'un poids moléculaire de 5000 à 10000.

Ein Inhibitor der Insulin-Wirkung am Muskel im Serum: I. Nachweis und Eigenschaften.

Zusammenfassung. 1. Die Messung der Wirkung intraperitoneal injizierten Insulins am Zwerchfell und am epididymalen Fettgewebe erlaubt den Nachweis von Inhibitoren der Insulinaktivität in der Injektionsflüssigkeit. 2. In einer 5%igen Lösung von Rinder-Albumin hat intraperitoneal injiziertes Insulin einen maximalen Effekt. 3. Die Insulinwirkung am Zwerchfell, nicht aber am Fettgewebe, wird signifikant vermindert, wenn das Hormon zusammen mit Seren von männlichen Ratten und Menschen injiziert wird. 4. Die Inhibitor-Aktivität befindet sich in der Albuminfraktion der betreffenden Seren und wird durch Dialyse des Serums oder der Albuminfraktion nicht vermindert. 5. Sie kann nach Sieden bei pH 4 bis 5 vom Albumin abgetrennt werden. Bei der inhibitorisch aktiven Substanz scheint es sich um ein Peptid mit MG zwischen 5000 und 10000 zu handeln.

Key-words: Insulin inhibitor, Serum insulin inhibitor, Muscle, Adipose tissue, Polypeptide.

Studies [14, 15, 16] on the hexose transport system in the isolated, perfused rat heart showed that there was a pronounced seasonal variation in the sensitivity of the tissue to insulin, with a maximum in summer and a minimum in winter. The dose-response curves relating the rate of sugar transport (efflux of galactose) to the insulin concentration were Michaelis-Menten in form for both summer and winter, and the appropriate graphical test showed that the reduced sensitivity in winter was explicable in terms of a competitive inhibitor of insulin action. Furthermore, the seasonal variation was found to be a photoperiodic effect, thus implicating the hypothalamic-pituitary axis in the phenomenon. In later work, not yet published, it was shown that the inhibition could be washed out of the tissue by increasing the contractile force of the heart. From an examination, in the light of these results, of seasonal changes in fat metabolism in the rat, and the photoperiodic control of lipogenesis in mammals and birds, it was suggested [16] that the inhibitor might be a hormone under hypothalamic-pituitary control, which promotes lipogenesis and metabolism of fat by restricting insulin action on muscle but leaving its action on adipose tissue unaffected. The first requirement for the substantiation of this hypothesis is the demonstration in the blood of an insulin inhibitor that is active on muscle but not on adipose tissue. Evidence for such a conclusion is presented in this paper.

Since the measurement of insulin action on both muscle and adipose tissue was required, the dual assay system of RAFAELSEN appeared ideal. RAFAELSEN [7, 8] showed that the intraperitoneal injection into the rat of small doses of insulin increased the glycogen content of the diaphragm, even when the amount injected was not enough to produce a significant effect on the blood glucose level. The injection of uniformly labelled glucose simultaneously with insulin, and the subsequent determination of the incorporation of the

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radioactive carbon into the glycogen of the diaphragm and into the lipids of the epididymal adipose tissue, was demonstrated to be a sensitive method for the assay of insulin-like activity on either tissue [9].

Methods

The intraperitoneal assay of insulin activity

The animals used were albino rats of Wistar stock bred in this laboratory. For the assay, weanling rats of both sexes weighing 60—100 g were used; the weight range for any given assay was never more than 20 g. The rats were fasted from 5 pm on the day prior to the assay, that is 18—20 h before killing. In this size male rat it was found that the suturing of the scrotal space [9], in order to insure good contact between the epididymal adipose tissue and the material injected into the peritoneal cavity, was not required. It sufficed to displace where necessary the testes into the peritoneal cavity at the commencement of the assay; the epididymal tissue thereupon remaining in the abdomen for the succeeding two hours.

Into each animal was injected intraperitoneally 1.5 ml of fluid, 1.0 ml of which consisted of the serum under examination or a solution (5 g/100 ml) of bovine serum albumin (Behringwerke AG., Marburg-Lahn, Germany), which had been extensively dialyzed against physiological saline (9 g/1000 ml). The remainder of the injected volume was physiological saline containing radioactive glucose, Evans Blue conjugated with albumin, and insulin when it was used. The radioactive glucose (Radiochemical Centre, Amersham, England) was uniformly labelled D-glucose-^{14}C; no difference was found between the preparation with 2—4 mc/mM and 50—150 mc/mM. One μc was injected into each rat. Evans Blue that was free of coloured impurities (British Drug Houses Ltd, Poole, England) was conjugated with bovine plasma albumin (20 mg of dye, and 490 mg of albumin in 100 ml of saline), dialyzed for 24 h against physiological saline, and the pH adjusted to 7.4. Of this solution 0.2 ml was injected into each rat to ascertain that the injected material went into the peritoneal cavity and not into the intestine or subcutaneously [12]. In unanaesthetized rats this precaution is not really necessary as such mishaps are rare (2 instances of the injected material going into the intestine in 4000 rats). Crystalline insulin, 23.5 U/mg, (Eli Lilly and Co., Indianapolis, U.S.A.) was used; a stock solution (1 U/ml) being diluted with physiological saline containing gelatine (200 mg/100 ml). The insulin concentrations used ranged from 250 to 4000 μU/ml of injected fluid; Rafaelsen having shown previously that doses of insulin as high as 10000 μU had no effect on the blood sugar [7].

The assay procedure was as follows: the rats were given the intraperitoneal injection in succession, and killed in the same order 2 h later. The time interval between successive rats was 3 min if only the diaphragm was to be excised, and 4 min if both the diaphragm and the epididymal or parametrial adipose tissue were required. The rats were anaesthetized with a gas mixture of 50% CO_2 : 50% O_2, which takes about 45 sec., decapitated, the abdomen opened and the tissues excised. The diaphragm was rinsed in ice-cold physiological saline, blotted, frozen in acetone containing solid carbon dioxide and weighed on a torsion balance. The tissue was then put into hot 30% potassium hydroxide (1 ml) and dissolved by heating for 10 min in a boiling water bath. After cooling, 0.4 ml of 2% aqueous sodium sulphate and 3.2 ml absolute ethanol were added with thorough mixing and the tubes kept at —20°C for at least 1 h. After centrifugation, the precipitate was washed with 2 ml of 66% aqueous ethanol, dried in a current of air and dissolved in 0.7 ml of water, 0.5 ml of which was taken for counting. Both pieces of adipose tissue from each rat were analysed together. After rinsing, they were weighed unfrozen and extracted for 12 h in 20 ml of chloroform: methanol (2 : 1). The tissue was then removed, and the solvent washed with 5 ml water, adjusted to 25 ml with more solvent, dehydrated by the addition of anhydrous sodium sulphate and a 10 ml sample evaporated to dryness in a counting vial at 60°C in a stream of air. Ten ml of toluene scintillation fluid (4 g PPO and 40 mg POPOP per 1000 ml toluene) was added to the vial and counted in a liquid scintillation counter (Tri-carb model 314 Ex-2, Packard Instrument Co., La Grange, Ill., U.S.A.). The glycogen was counted in the dioxane medium of BRAY [1]. Although a precipitate was formed with this medium, the samples could be counted satisfactorily, and no improvement was obtained by use of "Cab-o-sil" (Packard Instrument Co.).

Unless otherwise stated, all solutions used for intraperitoneal injection were dialyzed overnight against physiological saline. Sera and solutions of bovine serum albumin were dialyzed in Visking tubing No. 25/32. Solutions of polyvinyl pyrrolidone (mol. wt. 40000 and 150000), polyethylene glycol (mol. wt. 20000), dextran (mol. wt. 70000) (all from Fluka AG, Basel), and dextran (mol. wt. 70000; "Macrodex" 6%, Pharmacia, Uppsala, Sweden) were dialyzed in Visking tubing No. 23/32.

Serum was obtained from male rats of the same stock as those used in the assay, weighing 250—400 g, which had been fasted overnight. Blood was collected from the animals under ether anaesthesia, either by decapitation or by cardiac puncture, allowed to stand for two hours at room temperature and then centrifuged.

Free, that is nonësterified, fatty acids of the sera and of solutions of bovine serum albumin were determined by the method of DOLE and MEINERTZ [3].

Preparation of serum albumin

Serum albumin was prepared by two methods. The first, which was used only for rat serum, was the method of DEBRO et al. [2], in which advantage is taken of

the solubility of serum albumin in ethyl alcohol acidified with trichloracetic acid, whereas the other serum proteins are precipitated. The second method, which was used for both rat and pig serum, was that of RICHTER [11] in which serum albumin is selectively precipitated with a quaternary ammonium compound; the particular advantage of this method being that in contrast to other methods the albumin is the first species to precipitate. To a solution of 30 ml of serum (pH 7.5) in 59 ml of distilled water was added 2 ml of 17.5% (w/v) solution of hexadecyl pyridinium bromide (Fluka AG, Basel). The pH was adjusted to 8.6 using 1.0 NaOH. The mixture was warmed to 40—42°C and allowed to remain at this temperature for 30 min before centrifugation. The precipitate was "dissolved" in 40 ml of 0.01 N HCl by vigorous mixing with a magnetic stirrer for 30 min. The opalescent solution was then dialyzed in Visking tubing (No. 25/32) against N/200 HCl (pig serum albumin) or N/200 HCl in physiological saline (rat serum albumin) for 6—8 days in a dialyzing unit of the type described in the appendix to this paper. If distilled water was used with rat serum albumin the contents of the sac set as a gel, though this was readily reversible.

The data are presented as the mean ± standard error of the mean, and in parentheses the number of receptor rats used. In experiments where the incorporation of radioactivity was appreciable in the absence of added insulin (eg. with serum), the sums of the squared deviations (Σx^2) of the data in the presence and absence of insulin were added in calculating the S.E. of the mean insulin effectiveness.

Results

In adapting the intraperitoneal assay to the detection of the serum inhibitor, it was first necessary to select an injection medium for the insulin standard. Ideally, the medium chosen should contain no inhibitory material, and should permit the maximum insulin response. That these requirements are not necessarily the same, is evident from the data obtained for physiological saline, which although it contains no inhibitory material certainly did not give the maximum response (Fig. 1). A 5% bovine serum albumin solution in physiological saline was selected as a possible standard, and all other solutions compared with it. The first comparison was made with physiological saline alone. Saline and 5% albumin solutions, with and without insulin (1000 μU/ml) were injected intraperitoneally according to the assay procedure, and the incorporation of radioactive carbon into the glycogen of the diaphragm and into the total lipids of the epididymal fat pad determined. It was found that while on the adipose tissue the effectiveness of the insulin was the same for both solutions, on the diaphragm it was much less for the saline (56% of the value obtained for albumin). To establish this difference with greater certainty, dose response curves for insulin injected in physiological saline were obtained for both the diaphragm and the epididymal fat pads, and compared with the response to the median dose of insulin given in albumin (such dose response curves for insulin in 5% albumin were reproducibly linear, see Fig. 4). The data, which are illustrated in Fig. 1, confirm that the insulin effect

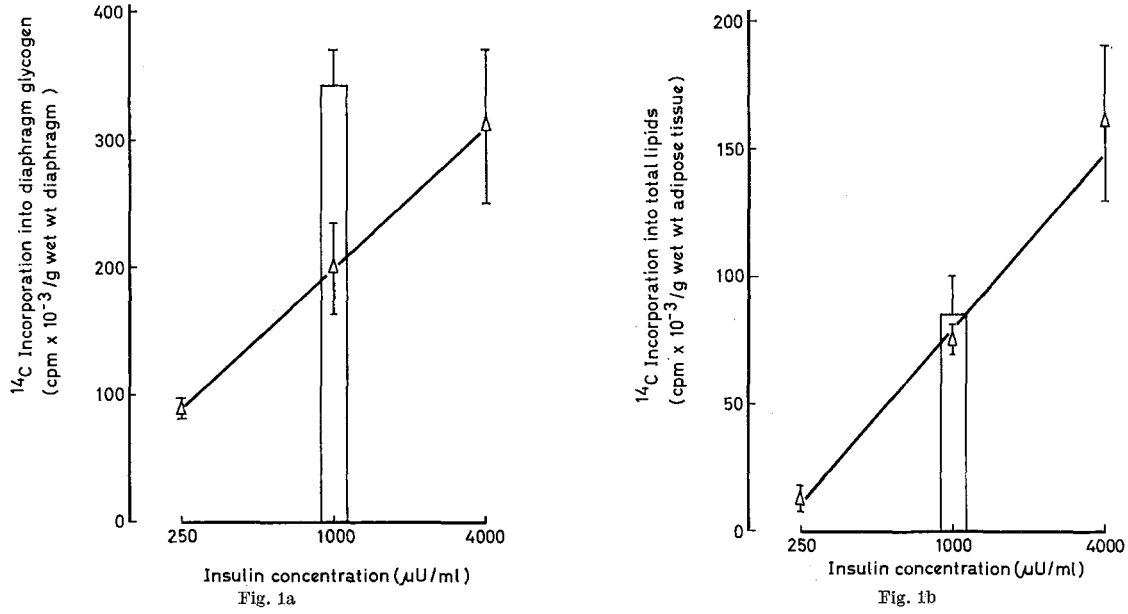

Fig. 1a Fig. 1b

Fig. 1. Comparison of the dose-response curves of insulin injected in saline (0.9%) with effects of the median dose of insulin in 5% bovine serum albumin on incorporation of glucose-u-^{14}C into: (a) diaphragm glycogen (b) total lipids of epididymal adipose tissue; △ insulin in saline; the bars, insulin in albumin (1000 μU/ml). Points and bars represent means of 8 rats ± S.E.

on the diaphragm is increased by the presence of albumin, but not the insulin effect on the adipose tissue. The incorporation, in the absence of insulin, of radioactive carbon into diaphragm glycogen was the same for both the saline and 5% albumin solutions, amounting to 1% of the effect of insulin at a concentration of 1000 μU/ml. With the epididymal tissue, the incorporation into total lipids was slightly higher for 5% albumin (14 $\pm$ 1%) than for saline (10 $\pm$ 1%).

The effectiveness of insulin (1000 μU/ml) injected intraperitoneally on the incorporation of radioactive glucose into glycogen of the diaphragm was determined for a range of albumin concentrations in physiological saline, and expressed as a percentage of the effect in 5% albumin (Fig. 2). Saline alone permitted the least insulin activity (57%). The effect of albumin was evident at 0.15 mM (1 g/100 ml) and the maximal effect was obtained over the concentration range

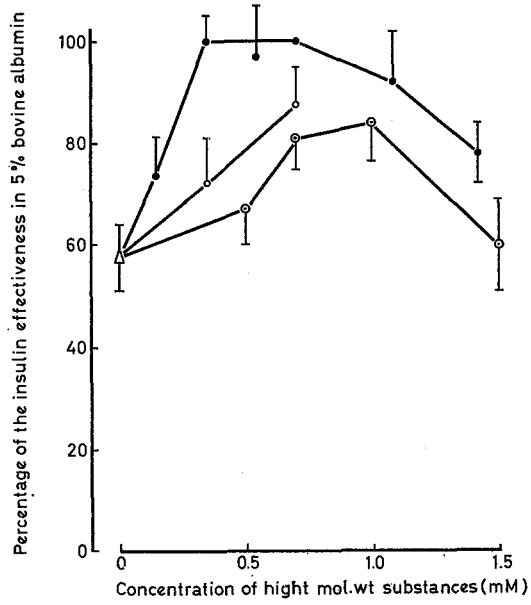

Fig. 2. Effectiveness of insulin (1000 μU/ml) on diaphragm (incorporation of glucose-u-^{14}C into glycogen) when given in saline solutions of high mol. wt substances, expressed as percent of effect of insulin (1000 μU/ml) in 5% bovine serum albumin in saline; ● bovine serum albumin; ○ polyvinyl pyrrolidone (mol.wt. 40000); ⊙ polyethylene glycol (mol. wt. 20000); △ saline. S.E. of ratio indicated for each point (7 rats), was calculated using the expression[18]:

$$\text{S.E.} = 100 \sqrt{\frac{R_e^2}{R_c^2}\left(\frac{S_e^2}{R_e^2} + \frac{S_c^2}{R_c^2}\right)}$$

where R_e and R_c are the mean values for exp. and control groups respectively, and S_e^2 and S_c^2 are variances of same.

0.35—0.75 mM (2.5—5.0 g/100 ml). At higher concentrations there was a decrease in effect.

Binding of the insulin to albumin or the slower absorption of the fluid from the peritoneal cavity due to the colloid osmotic pressure are two possibilities that could account for the difference between saline and albumin solutions, and both these factors might be expected to be of greater consequence for the diaphragm because of its position in the peritoneal cavity. The binding of insulin would appear the less likely because of the considerable increase in effect of 2.5% compared with 1.0% albumin, whereas the latter concentration should satisfactorily bind much greater quantities of insulin than are involved here. If the effect merely reflects the colloid osmotic pressure of the solution it should not be specific for albumin, and the injection of other high molecular substances dissolved in physiological saline should have the same effect. Accordingly two preparations of dextran (mol. wt. 70000) were used at 0.75 mM (5 g/100 ml). The first (Fluka AG, Basel) caused inflammation of the peritoneum, and a reduction in insulin effectiveness was observed (37 $\pm$ 5% (7) of the effect in 5% albumin). The experiment was repeated using another preparation of dextran of the same molecular weight (Macrodex 6%. Pharmacia, Uppsala, Sweden) purchased as a sterile solution for intravenous injection. Although this preparation did not cause an inflammatory reaction or ascites, it gave an even greater reduction in insulin effectiveness (16 $\pm$ 4% (7) of the effect in 5% albumin). A much higher molecular weight polymer, polyvinyl pyrrolidone (mol. wt. 150000) did give an increase insulin response (71 $\pm$ 8% (7) at 0.7 mM) compared with saline; however, it was significantly less than albumin (all these comparisons were with 5% albumin and the results are expressed as ratios of the insulin effect in the latter solution). Two synthetic polymers with molecular weights less than that of albumin were then examined: polyvinyl pyrrolidone (mol. wt. 40000) and polyethylene glycol (mol. wt. 20000). Both gave an increase in the effectiveness of insulin compared with saline alone; the former was probably the more effective, giving at 0.7 mM 87% of the effect in 5% albumin, whereas the optimal effect of the polyethylene glycol was 83% at 1.0 mM (Fig. 2). As with albumin, a higher concentration of polyethylene glycol (1.5 mM) gave a decrease in effect. Further evidence supporting the proposition that the effect of the albumin is due to its colloid osmotic pressure, was obtained in the following experiment. A solution containing both albumin (0.15 mM) and polyvinyl pyrrolidone (mol. wt. 40000; 0.35 mM) was compared with 5% albumin. The insulin effectiveness in the former was found to be 87 $\pm$ 9% (6) of that in the latter, a substantial increase over the percentages found with either component alone at those concentrations (Fig. 2).

Precision and reproducibility of, and the effect of pH on, the intraperitoneal assay of insulin activity on the diaphragm.

The standard deviation of the ^{14}C incorporation into diaphragm glycogen was calculated for all the animals receiving the standard insulin solution (1000 μU/ml in 5% albumin) in 30 assays. The value expressed as a percentage of the insulin effect was 24%. A measure of the reproducibility of the assay is given by the com-

parison, in the same assay, of 4 groups each of 6 rats injected with the standard insulin solution (1000 μU/ml in 5% albumin). The values for the ^{14}C incorporation, expressed as counts per minute $\times 10^{-3}$/g wet wt. of diaphragm, were for the 4 groups: 218 ± 37; 214 ± 21; 220 ± 25; and 211 ± 20.

The solution of bovine serum albumin (5 g/100 ml) used as the injection medium for the standard dose of insulin, had a pH of 6.8. To determine whether slight variations in pH in excess of 7.0 affected the activity of insulin on the diaphragm, the incorporation of ^{14}C into diaphragm glycogen was determined for insulin (1000 μU/ml) given in two 5% albumin solutions, one at pH 6.8 and the other at pH 8.0. The value for the incorporation in the former was 113 ± 18 (6) cpm $\times 10^{-3}$/g wet wt.; for the latter, 115 ± 15 (6).

The presence in serum of an inhibitor of insulin action on muscle

Rat serum Blood was obtained from normal, fasted (15 h), male rats by cardiac puncture under ether anaesthesia. After standing for 3 h, the serum was separated by centrifugation and injected with and without insulin (1000 μU/ml) into small rats according to the procedure of the intraperitoneal assay of insulin activity. The incorporation of ^{14}C into glycogen of the diaphragm and total lipids of the epididymal adipose tissue was determined for the serum and compared with that for insulin given in 5% bovine serum albumin (Fig. 3). It is evident from the data illustrated in this figure that insulin was very much less effective on the diaphragm when given in serum than when given in 5% albumin, whereas there was no difference on the adipose tissue.

However, as the incorporation of radioactivity into the diaphragm glycogen in the absence of insulin was so much greater with serum than with albumin, it is possible that the effectiveness of the added insulin is less only because the maximum response of the diaphragm has been reached. Although this is unlikely, since RAFAELSEN et al. [9] showed that the maximum response was certainly not obtained with a dose of insulin less than 10000 μU, this important possibility was ruled out by comparing the dose-response curves of insulin added to the serum and to the bovine albumin solution (Fig. 4). There was a very significant reduction in the effectiveness of all three doses of insulin on the diaphragm when the hormone was injected with serum, whereas there was no such difference on the adipose tissue.

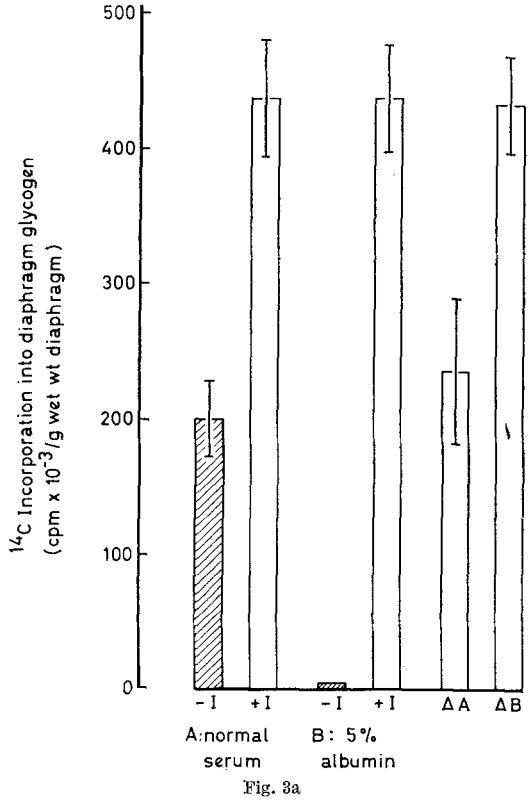

Fig. 3a

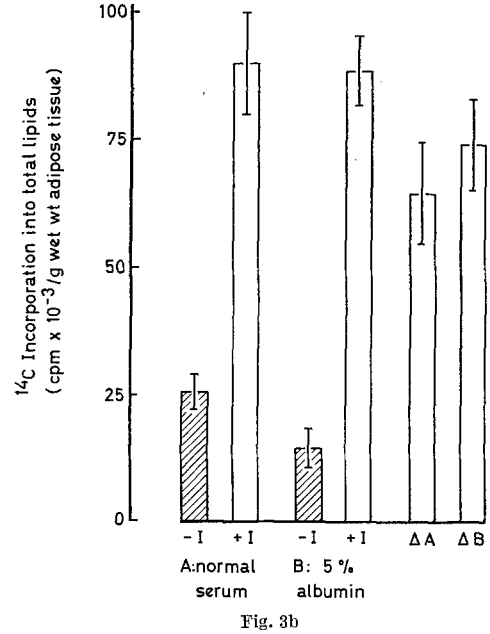

Fig. 3b

Fig. 3. Effectiveness of insulin (1000 μU/ml) injected in 5% bovine serum albumin and in serum from fasted male rats on incorporation of glucose-u-^{14}C into: (a) diaphragm glycogen (b) total lipids of epididymal adipose tissue. Each bar represents mean of 5 rats $\pm$ S.E.; differences between values with and without insulin given by last 2 bars on right.

Another possibility is that the insulin effectiveness should not be assessed by simple subtraction when the quantities concerned are related in a logarithmic manner. The evidence against the application of this argument to this situation will be given in detail in a later paper [17], but it may be summarized as follows; 1. the measured insulin-like activity of the serum, except for that from fed animals, is almost exclusively non-suppressible, and its effect is arithmetically additive to that of added crystalline insulin; 2. levels of endogenous insulin-like activity comparable with those found in the serum of normal male rats are present in sera that do not contain inhibitory activity (eg. from hypophysectomized rats, 3-day fasted rats, female rats in oestrus, guinea pigs and sheep); and 3. inhibitory activity can be released *in vivo* without any change in the insulin-like activity of the serum.

In subsequent experiments the same results were found for serum from blood obtained by decapitation of the rats under ether or nembutal anaesthesia, for serum kept frozen at −20°C for 4 days, and for serum that had been freeze-dried. It has been reported that an inhibitor of insulin action on the rat hemidiaphragm can be detected in otherwise inactive solutions following dialysis [6, 4]. For this reason the effect of dialysis on the inhibitory activity of serum was specifically examined. The data of two experiments using different samples of serum and different concentrations of insulin (500 and 1000 μU/ml) are shown in Fig. 5. It is clear that dialysis for 24 h did not affect either the endogenous insulin-like activity of the serum or its capacity to reduce the effectiveness of added insulin.

Human serum. Blood samples were obtained from 8 normal male subjects (aged 30—40 years) who had fasted overnight, and the dialyzed pooled serum was injected intraperitoneally with and without insulin (250, 500 and 1000 μU/ml), according to the assay procedure. The dose response curves of the incorporation of ^{14}C into glycogen of the diaphragm and total lipids of the epididymal adipose tissue were determined for insulin given in the serum and in 5% bovine serum albumin solution (Fig. 6). As with the male rat serum, it is evident that insulin is much less effective on the diaphragm when given in human serum than when given in 5% albumin, whereas there was no difference on the adipose tissue.

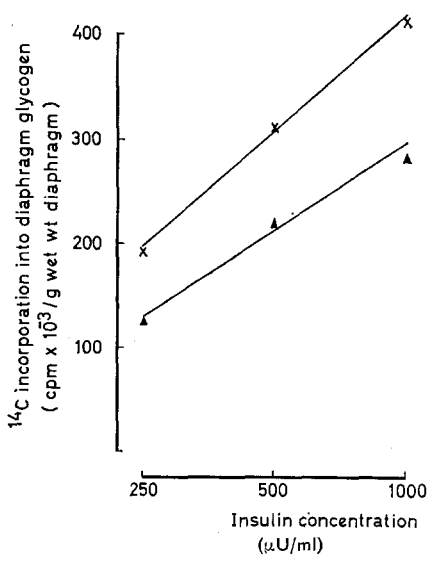

Fig. 4a

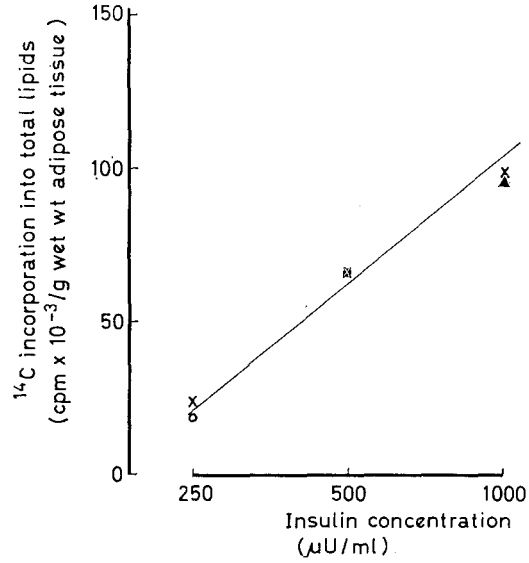

Fig. 4b

Fig. 4. Dose-response curves of insulin injected in 5% bovine serum albumin (×) and in serum from fasted male rats (▲) on incorporation of glucose-u-^{14}C into: (a) diaphragm glycogen (b) total lipids of epididymal adipose tissue. Each point represents mean of 12 rats.

Blood samples were also obtained from 5 male, maturity-onset diabetics who had fasted 18 h. Diabetes in these patients was controlled by dietary measures only, and none of them had ever received injections of insulin. The dialyzed pooled serum was injected with and without insulin (250, 1000 and 4000 μU/ml), and the comparison made with insulin injected at the same doses in 5% bovine serum albumin (Fig. 7). The results were the same as those obtained with sera from normal male subjects and male rats; that is, on the diaphragm the insulin effectiveness was less in the serum than in the albumin solution, whereas on the epididymal adipose tissue there was no difference.

Sera from other species. Samples of blood were obtained from males (in the first 3 species, castrated males) of the following species: pig (fasted 12 h), ox, sheep, mouse (Swiss albino) and guinea pig (fasted 12 h). The dialyzed sera of these species were injected in-

traperitoneally with and without insulin (1000 μU/ml) and the effectiveness of the insulin on the diaphragm compared with that of the same dose of insulin injected in 5% bovine serum albumin. Expressed as a percentage of the effect of insulin given in the bovine albumin solution, the results (mean $\pm$ S.E., no. of assay rats and the P value) were: pig, $47.5 \pm 12.4\%$ (6), $P < 0.03$; ox, $47.4 \pm 9.5\%$ (7), $P = 0.001$; sheep, $87.0 \pm 12\%$ (7), N.S.; mouse, $55.3 \pm 10.5\%$ (7), $P < 0.01$; and guinea pig, $116.3 \pm 13.1\%$ (7), N.S.

Free (nonësterified) fatty acids and insulin inhibition

As it has been suggested that free fatty acids have a role in reducing the sensitivity of muscle to insulin [10], the concentration of free fatty acids was determined for the serum (from fasted male rats) and the 5% bovine serum albumin that were used in the experiment shown in Fig. 4. The concentration in the serum was 920 μeq./l, and that in the albumin 1500 μeq./l. In another group of experiments, which are reported in detail in a later paper of this series [18], it was found using sera from 15 h fasted rats, that

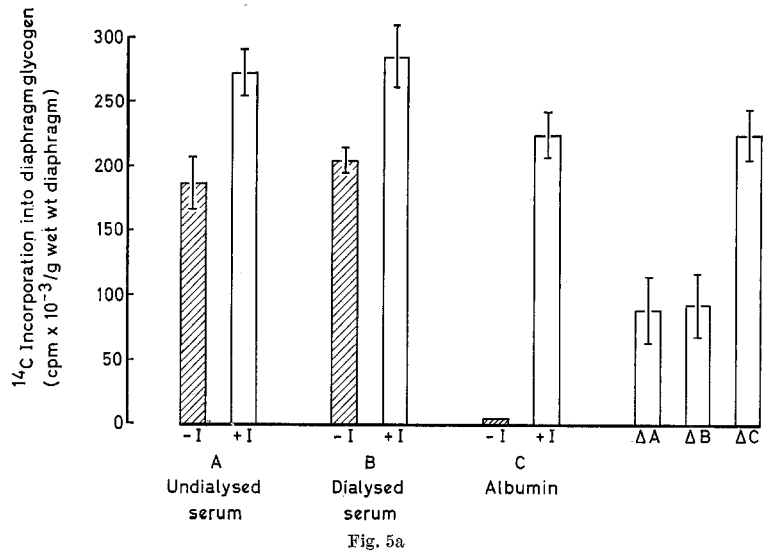

Fig. 5a

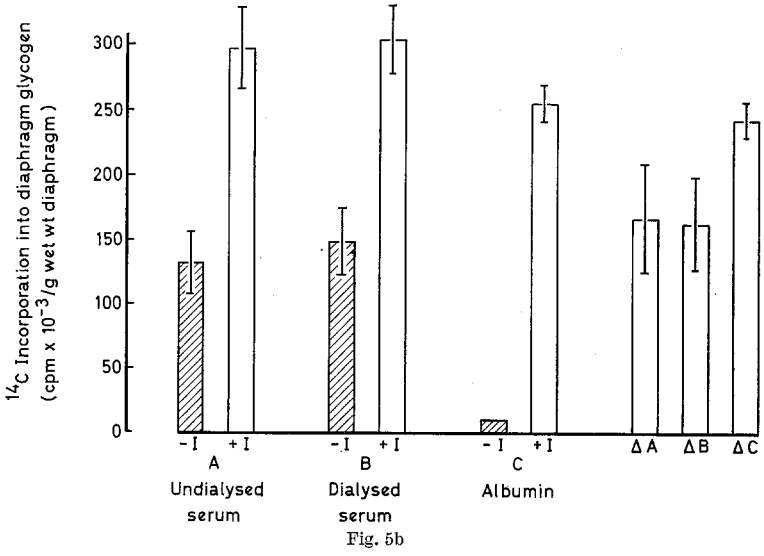

Fig. 5b

Fig. 5. Comparison of effectiveness of insulin (a) 500 (b) 1000 μU/ml injected in rat serum dialyzed overnight, in rat serum not dialyzed, and in 5% bovine serum albumin on incorporation of glucose-u-^{14}C into diaphragm glycogen. Each bar represents mean of 5 rats $\pm$ S.E.; differences between values with and without insulin given by last 3 bars on right.

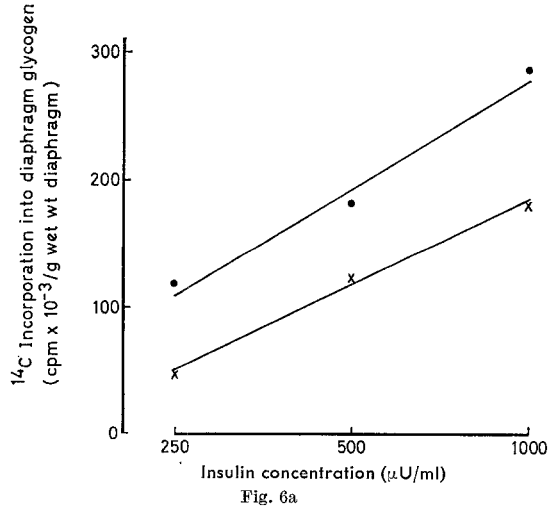

Fig. 6a

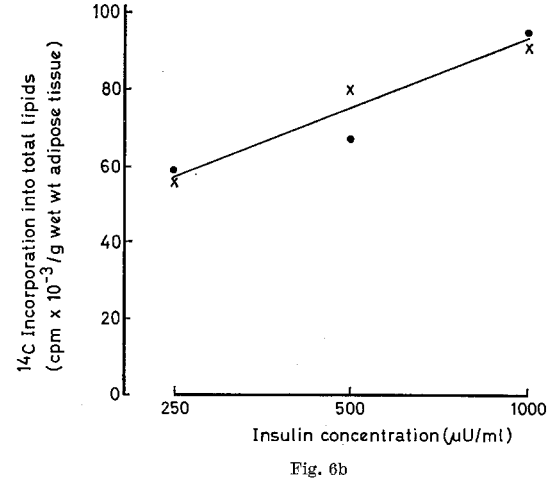

Fig. 6b

Fig. 6. Dose-response curves of insulin injected in 5% bovine serum albumin (●) and in serum from normal male subjects (×) on incorporation of glucose-u-^{14}C into; (a) diaphragm glycogen (b) total lipids of epididymal adipose tissue. Each point represents mean of 12 rats.

whereas the serum from normal males reduced the effectiveness of insulin as has been described above, the serum from hypophysectomized males and that from females in oestrus did not. Determination of the free fatty acids gave the following values per litre: 5% bovine albumin, 1200 μeq.; serum from normal males, 850 μeq.; from hypophysectomized males, 700 μeq.; and from females in oestrus, 900 μeq. There is obviously no correlation of insulin inhibition with concentration of free fatty acids in the injected solutions, which is in agreement with the finding obtained for the isolated diaphragm [5].

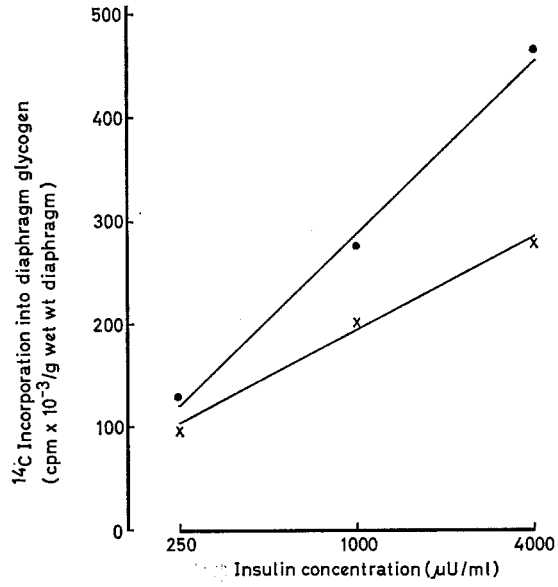

Fig. 7a

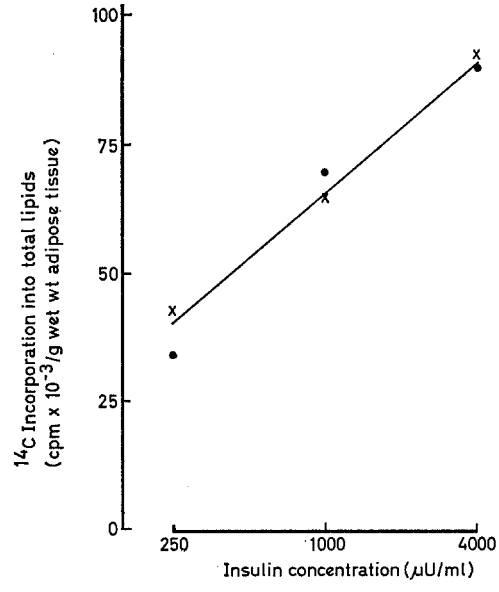

Fig. 7b

Fig. 7. Dose-response curves of insulin injected in 5% bovine serum albumin (●) and in serum from male, maturity-onset diabetics (×) on the incorporation of glucose-u-^{14}C into: (a) diaphragm glycogen (b) total lipids of epididymal adipose tissue. Each point represents mean of 6 rats.

Extraction of the inhibitor from serum

The first step in the isolation of the inhibitor was to determine with which fraction of the serum proteins the inhibition was associated. The albumin fraction of one half of a batch (80 ml) of pooled serum from fed male rats was prepared by the method of DEBRO et al. [2]; and the insulin inhibitory activity of the albumin, dissolved in 40 ml of physiological saline, compared with that of the remainder of the original serum using the intraperitoneal assay (Fig. 8). It is clearly evident that there was an appreciable reduction in insulin activity with the rat albumin, and furthermore that this reduction was approximately the same as that obtained with the original serum. Thus most, if not all of the inhibitory activity of the serum is associated with the albumin fraction.

However, the inhibitory activity cannot be a property of the albumin molecule itself, for the bovine serum albumin used as the standard injection solution in these experiments was without activity. This albumin was prepared by ethanol fractionation at low temperatures, and presumably since bovine serum was active in inhibiting insulin, this fractionation procedure leads to a dissociation of the inhibitory activity from the albumin. This is supported by experiments performed with human plasma albumin (Swiss Red Cross, Bern) prepared in the same way. Insulin (1000 and 4000 μU/ml) injected in a 5% solution of the human albumin gave 95 $\pm$ 9.6% (5) and 82 $\pm$ 19% (5)

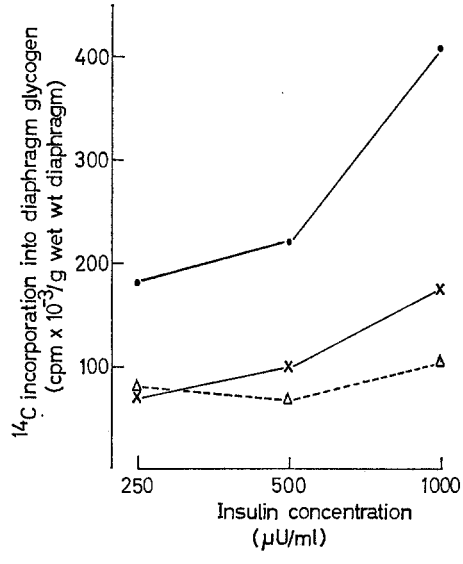

Fig. 8. Dose-response curves of insulin injected in 5% bovine serum albumin (●), in serum from fed male rats (×) and in albumin prepared from the same serum (△) on incorporation of glucose-u-^{14}C into diaphragm glycogen. Each point represents mean of 6 rats

of the insulin effectiveness in bovine serum albumin. This lack of an inhibitory effect should be compared with the very significant inhibition obtained with human serum (Fig. 6).

Boiling a saline solution of rat serum albumin for 10 min at pH 4—5 led to the inhibitory activity passing into the supernatant fluid, which was separated by centrifugation and freeze-dried. The solid was dissolved in a small volume, dialyzed against distilled water for 2 h in Visking tubing (No. 23/32) to remove the salt, freeze-dried and finally dissolved in 5% bovine serum albumin for intraperitoneal injection. This solution when tested in the intraperitoneal assay reduced very significantly the effectiveness of added insulin (1000 μU/ml): $43 \pm 5\%$ (6) of the effectiveness in 5% albumin alone.

There were two main drawbacks to the continued use of the trichloracetic acid-ethanol extraction for the preparation of serum albumin: first, the volume of extract was twenty times that of the original serum, which made the handling of even moderate quantities difficult; and secondly, a fat emulsion formed during dialysis that was difficult to remove, although this is certainly much less of a problem with serum from fasted rats. For these reasons another method of preparing albumin from serum, by precipitation with hexadecyl pyridinium bromide [11], was investigated in the hope that it could be used for larger quantities. Albumin prepared by this method (see Methods) may be somewhat altered in its properties; however, if sufficiently well dialyzed it will precipitate upon boiling for 10 min at pH 4—5 in saline solution, yielding a supernatant fluid with inhibitory activity comparable

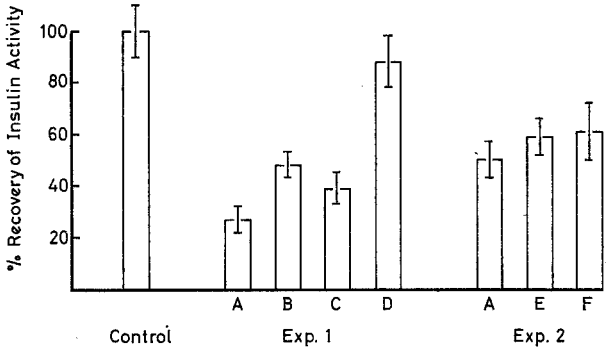

Fig. 9. Comparison of effectiveness of insulin (1000 μU/ml) on diaphragm (incorporation of glucose-u-^{14}C into glycogen) when given in 5% bovine serum albumin (control, 100%) with that in same solution but containing various preparations (A-F) of the serum inhibitor prepared by heat-denaturation of rat serum albumin. Each bar represents mean ± S.E. (6 rats). Exp. 1 Inhibitor was : (A) untreated; (B) adsorbed onto oxycellulose and eluted with 0.1 N HCl; (C) dialyzed in Visking tubing No. 23/32 for 24 h; (D) precipitated with equal vol. 5% trichloracetic acid. Exp. 2 Inhibitor was: (A) untreated; (E) boiled in 0.1 N HCl for 20 min; (F) passed through DEAE-cellulose (Cl^- form), pH 3.5

with that obtained from the original serum. Using a dialyzing unit of the type described in the appendix, it is possible to prepare in a single batch the albumin, which possesses inhibitory activity, from 1 litre of pig serum, thus providing in sizeable amounts a ready source of the inhibitor. Using heat-denatured extracts of such preparations of albumin from rat serum, the following properties have been observed.

Chemical. Although the inhibitor was quite stable in acid solution, and in fact could be boiled for 20 min in 0.1 N HCl without loss of activity, it was quickly inactivated at alkaline pH's, eg. pH 10 and over. It appears to be susceptible to oxidation, for the freeze-dried extracts of heat-denaturated albumin lost their activity when left for 3 days at 2°C. Other properties are indicated by the data shown in Fig. 9.

Physical. A solution of the heat-denaturated extract in physiological saline was only slightly inactivated by prolonged (12 h) dialysis in Visking tubing No. 23/32 and the same result was obtained with dialysis of inhibitor that had been boiled in 0.1 N HCl for 20 min. This would indicate that the molecular weight is 5000 or greater, a fact more definitely ascertained by fractionation of the extracts by gel filtration

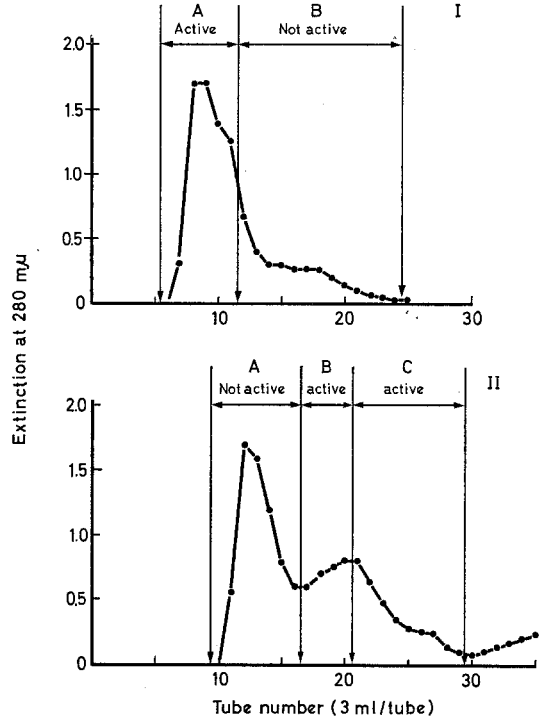

Fig. 10. Partial fractionation by gel filtration of extracts of heat-denatured albumin from pig serum, using Sephedex G-25 and G-50 equilibrated with acetic acid (pH 3.7). I. Column of G-25 (10 g), 2.0 × 15.0 cm, sample vol. 1.5 ml, flow rate 30 ml/h. II. Column of G-50 (10 g), 2.5 × 19.0 cm, sample vol. 1.5 ml, flow rate 20 ml/h. Eluate samples combined to give Fractions A, B, and C as shown : Fraction A containing in each case the material excluded from the gel particles. Inhibitory activity assessed by reduction in insulin effectiveness on diaphragm (incorporation of glucose-u-^{14}C into glycogen) when freeze-dried fractions were given in 5% bovine serum albumin containing insulin (1000 μU/ml)

on Sephedex G-25 and G-50 (Pharmacia, Uppsala, Sweden), as shown in Fig. 10. The samples put onto the column were both approximately equivalent to 70 ml of serum. The volume of eluate corresponding

to the material excluded from the gel particles had been determined for both columns using albumin, and the samples of eluate were accordingly pooled to give fractions corresponding to material of molecular weight above and below the approximate exclusion limits, based on polysaccharides, for the two gels (G-25, 5000; G-50, 10000). Each of the fractions was freeze dried and the inhibitory activity assessed by dissolving the solid in 5% bovine serum albumin containing insulin (1000 μU/ml), and expressing the data as a percentage of the insulin effectiveness of the control. It was found that Fraction A from the G-25 column was highly active (insulin effectiveness was $12 \pm 3\%$ (6)) whereas Fraction B was devoid of activity. With the G-50 column, Fraction A was almost devoid of activity ($83 \pm 11\%$ (6)), whereas Fractions B and C were very active ($26 \pm 5\%$ (6) and $21 \pm 10\%$ (6)).

Biological. The dose response curve was obtained for the inhibitor prepared by heat denaturation of rat serum albumin. The response measured was the effectiveness of insulin (1000 μU/ml) on the diaphragm in the presence of various concentrations of the inhibitor, the values being expressed as a percentage of the insulin effectiveness in the bovine albumine alone.

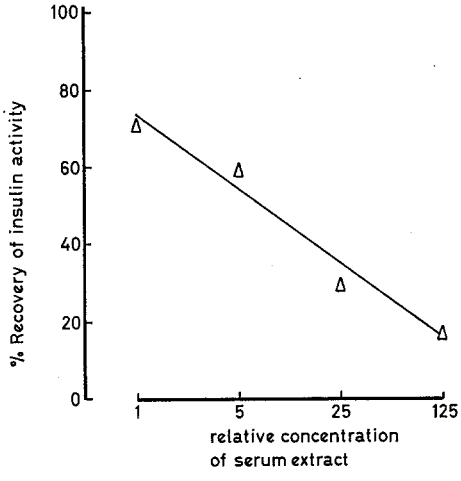

Fig. 11. Dose-response curve for the serum inhibitor prepared by heat-denaturation of rat serum albumin. Response measured as percent recovery of effectiveness of insulin (1000 μU/ml) on diaphragm (incorporation of glucose-u-^{14}C into glycogen) in 5% bovine serum albumin when various quantities of the freeze-dried extract were dissolved in the solution. Each point represents mean value for 6 rats.

Preparation of the inhibitor by heat-denaturation of the whole serum

Because of the possible production of artefacts in the process of preparing the albumin fraction from serum (more especially by the trichloracetic acid-ethanol method), it seemed desirable to investigate the possibility of preparing the inhibitor by heat-denaturation of the whole serum. Serum from male rats was diluted with an equal volume of physiological saline, adjusted to pH 4.5 and boiled for 10 min. After cooling, the solution was filtered, concentrated in a rotary evaporator and dialyzed (Visking tubing No. 23/32) for 2 h against distilled water to remove the salt. The dialyzed solution was freeze-dried, and the solid dissolved in 5% bovine serum albumin, which was injected intraperitoneally with and without insulin (1000 μU/ml). The resulting insulin effectiveness was expressed as a percentage of that in bovine serum albumin alone. The insulin-like activity of the heat-denatured extract of the serum, without any added insulin, amounted to 70% of the control (i.e. bovine albumin with insulin), and it would be more satisfactory if the endogenous insulin-like activity of the extract could be reduced or eliminated. To secure this end two procedures were tried. In the first, a sample of the heat-denatured extract of serum was acidified (pH 3.5) with HCl and passed through a column of DEAE-cellulose (Cl^- form) equilibrated at the same pH. The eluate showed a much reduced level of insulin-like activity ($11 \pm 3\%$ (6) of the control), and when insulin (1000 μU/ml) was added its effectiveness was significantly less ($59 \pm 11\%$ (6)) than in the control. The second procedure utilized the adsorption of the inhibitor by oxycellulose. After shaking the heat-denatured extract of serum in 0.1 N acetic for 24 h with oxycellulose, the latter was extracted with 0.1 N HCl, and the resulting extract neutralized, dialyzed and freeze-dried. The solid when dissolved in 5% bovine albumin had no insulin-like activity (the insulin-like effect was 1%, and that of the albumin alone 1.5% that of the control); but when insulin (1000 μU/ml) was added to it, only $53 \pm 11\%$ (6) of the control effect was obtained.

In none of these procedures using whole serum was the yield of inhibitor comparable with that obtained by heat-denaturation of the serum albumin, and for this reason these experiments are only of interest in that they show that the inhibitor is not an artefact arising from the preparation of the albumin.

Discussion

The demonstration presented in this paper of an insulin inhibitor in the whole untreated serum from normal rats and normal human subjects is the first to the author's knowledge. That the inhibitor is active on muscle but not on adipose tissue satisfies the essential requirement of the hypothesis, discussed in the introduction, that the relative importance of fat and carbohydrate metabolism is controlled by an insulin inhibitor active only on muscle. If the inhibitor is an endocrine factor with such a physiological role, then its demonstration in serum is extremly relevant to the suggestion of many [19] that an insulin antagonist contributes to the pathogenesis of diabetes mellitus.

As regards the demonstration of the inhibitor in extracts of serum, first in the albumin fraction and subsequently in protein-free solutions, one must of

course be more cautious. Probably many polypeptides will inhibit insulin action on muscle, and the problem is to be certain that the inhibitory activity measured in the extracts is due to the original inhibitor and not due to an artefact produced in the extraction process. One way of approaching this problem is to prepare the inhibitory activity by a variety of procedures, and the results of such an attempt have been described. Certainly the preparation of an active albumin fraction by another procedure than the trichloracetic acid-ethanol method was a significant improvement. Finally, it is important to note that subsequent experiments investigating the physiological role of this serum inhibitor of insulin have been performed with whole serum [17], thus avoiding the very real doubts pertaining to extracts of serum in this regard.

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Appendix

An apparatus for the more efficient dialysis of moderate volumes of fluid

The experiments described in the foregoing paper necessitated the prolonged (8 days), thorough dialysis of relatively large volumes (500 ml) of solutions of albumin. The following apparatus, which was constructed by Mr J. MOLLARD, our fine-instrument mechanic, incorporates in a convenient form an obvious means of improving dialysis. The apparatus consists essentially of a bath in which dialysis sacs can be slowly rotated at an angle to the horizontal; thus permitting the continuous mixing of not only the dialyzing fluid, but also, by the inclusion of glass or stainless-steel balls in the sacs, the fluid to be dialyzed. After developing the final form of the apparatus, we found that the same basic principle had been used in a design published earlier [1].

The apparatus is constructed completely in polyvinyl chloride (PVC) and cemented with PVC cement ("Somothyl", Soc. pour Métaux ouvrés, Geneva). The inside dimensions[1] of the bath, which is constructed in PVC sheeting (*t* 0.5 cm), are: *l* 45 cm; *w* (top) 18 cm; *h* (max) 19 cm; and it had a capacity of 6 litres minimum, and 8 litres maximum. The sacs are rotated by means of an axle (*d* 2 cm; *l* 45 cm), on which are mounted 3 wheels as shown in the accompanying figures. Two are permanently fixed at one end; the outer one (*d* 7.5 cm; *t* 2 cm) is grooved to take a nylon pulley belt, which is connected to a motor mounted 20 cm above the apparatus; the inner wheel (*d* 12 cm; *t* 0.5 cm)

[1] *t*, thickness; *l*, length; *w*, width; *h*, height; and *d*, diameter.

has 6 notches equally spaced around its circumference to accommodate the knotted ends of the dialysis tubing. The third wheel, at the opposite end, is held rigidly to the axle by a removable pin; since the axle is pierced every 5 cm to receive this pin, the distance between the wheels can be varied according to the length of the dialysis sacs used (12—38 cm). This movable wheel is constructed from 2 outer discs (*d* 13 cm; *t* 0.5 cm) cemented to a smaller inner disc (*d* 9 cm; *t* 0.25 cm) as indicated in the inset drawing. Again there are 6 notches around the circumference to correspond to the notches on the fixed wheel. The free end of the dialysis sac is clamped firmly in the notch by a hinged clip, which is then fastened by a pin (see inset). For loading, the axle is raised by means of the attached vertical arms (*l* 20 cm) to the top of the bath, where it rests in grooves, and is held in this position by means of the locking arms (Fig. 12). In this position the axle can still rotate though other movement is prevented. By means of a spring-loaded arm (not shown), the end of which can fit into slots on the exposed end of the axle, the latter can be immobilized at each of the six positions corresponding to the sacs. The sacs, each of which contains one or two stainless-steel ball-bearings, are mounted at an angle of 25° to the horizontal. When dialysis is in progress, the axle will rise under the tension of the pulley belt unless it is rigidly held in place. This is accomplished by removable pins inserted through both the outer casing and the vertical arms used for raising the axle. The axle is rotated at approximately 3 rev/min. A short length of polythene piping, which is terminated by a tap of the same material, extends from a hole in the bottom of the bath to a sink. In prolonged dialysis, deionized water rather than tap-water has been used for all but the final stages, when distilled water is essential.

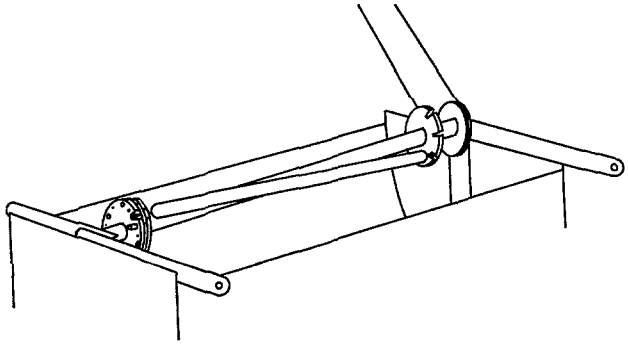

Fig. 12

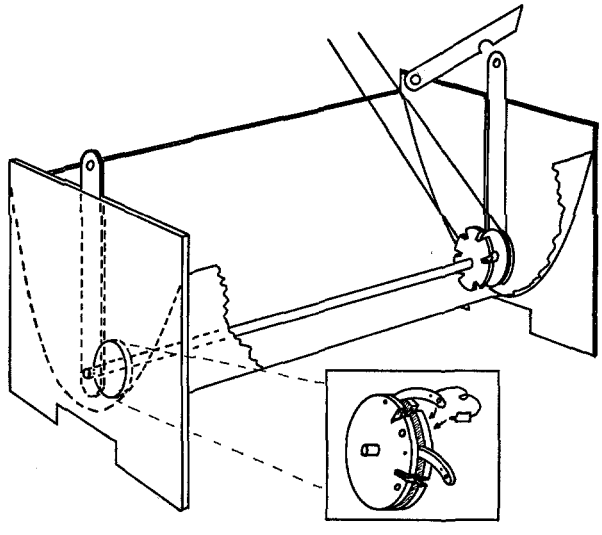

Fig. 13

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[1] Feinstein, R.N.: An apparatus for increasing the speed of dialysis of small quantities of fluids. J. Lab. clin. Med. **40**, 313—315 (1952).

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