# Insulin-like Activity by Fat Pad Assay and Immunoreactive Insulin

## II. Their Occurrence in Fractions by Gel Filtration of Serum

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SUMMARY

**In a previous report, serum insulin was extracted with efficient recovery by salt-ethanol extraction.**

**As the second method of separation of serum insulin gel filtration was adopted. Dog sera obtained from the pancreatic and the femoral veins were separated into two fractions (A and B) with Sephadex G-50. Fractions A and B corresponded respectively to the major peak of serum proteins and the portion where I-131-insulin added to serum was eluted. Insulin-like activity (ILA) was assayed by rat fat pad and immunoreactive insulin (IRI) by a double antibody method. Significant ILA was demonstrated in both fractions. IRI in serum appeared mostly in fraction B. ILA/IRI ratio was closer to 1.0 in fraction B than in serum. Fraction A tended to have fairly uniform ILA irrespectively of origin of serum, often exceeding the ILA of serum. Fraction A from a depancreatized dog serum had similar degree of ILA. Serum and both fractions stimulated various phases of fat pad metabolism similarly to insulin, including glycogen synthesis. Assays based on four different metabolic parameters gave essentially the same ILA values for both serum and fractions. ILA of fraction A differed from insulin in its nonneutralizability with anti-insulin serum, its poor suppressibility by cysteine, and the lack of parallelism of its regression line with that of insulin. Simple dilution and reconcentration of serum resulted in an increase in its ILA. Presumably, fraction B is primarily due to serum insulin with similar immunological and biological activities to pancreatic insulin. Whether or not ILA of fraction A is related to insulin remains unknown.** DIABETES *18*:75-83, February, 1969.

The nature of insulin is a matter of controversy.[1] Our preceding study demonstrated that extraction of serum by acid-ethanol or acid-ethanol-NaCl procedures did not affect serum immunoreactive insulin (IRI) but reduced considerably insulin-like activity (ILA) by fat pad assay.[2] Although there was still a tendency of higher ILA values than IRI in these extracts as in original sera, ILA approached IRI after extraction. The major part of ILA which is not immunologically reactive was eliminated by these extraction procedures.

In the present study, in order to analyze this ILA, separation of IRI from other serum proteins was attempted by gel filtration with Sephadex G-50. By this method serum was separated into two fractions, one of which consists of most serum proteins and contains scarcely any IRI (Fr. A), while the other supposedly contains endogenous insulin with the similar molecular size to the extracted pancreatic insulin (Fr. B). Concurrently with the radioimmunoassay for IRI determinations, the insulin-like activities of both fractions were tested on various parameters of rat fat pad metabolism including the suppression of the activities with anti-insulin serum and cysteine.

Dog serum was used in this study, in order to test both the peripheral vein and the pancreatic vein blood.

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### MATERIALS AND METHODS

*Blood samples*

Blood of dogs was withdrawn under anesthesia from the femoral vein and the superior pancreaticoduodenal vein by catheterization, at fasting and ten to thirty minutes after intravenous injection of 1.0 gm./kg. glucose. Serum was separated by centrifugation and kept

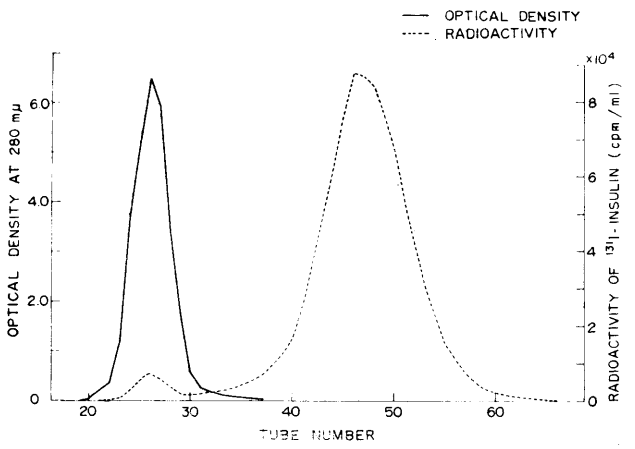


FIG. 1. Separation by a Sephadex column (G-50, coarse) of normal dog serum added with I-131-insulin (6-10 mc./mg.). Solid line represents the optical density at 280 mμ. of the eluate and dotted line represents radioactivity of I-131-insulin.

frozen at —20° C.

*Preparation of fractions*

Gel filtration was carried out according to the method of Flodin and Killander.[3] Sephadex G-50 (coarse, Pharmacia, Uppsala) was packed in a glass column (4 × 80 cm.) after being swollen in distilled water. Then 0.1 M Tris-HCl buffer in 0.2 M NaCl (pH 8.0) was run overnight through the column at 4° C. The final height of the gel bed was 60 cm.; 10 ml. serum was applied to this column, eluted with the same buffer at a flow rate of 75-80 ml./hr. and collected in 10 ml. portions by a fraction collector. Whenever a new column was packed, 10 ml. of serum which had been incubated overnight at 4° C. with 10 μg. of I-131-insulin (Abbott, 6-10 mc./mg.), was run at first, and the optical density at 280 mμ. and the radioactivity of the eluate were determined. There was only very little overlapping between the peak of major serum proteins and the peak of radioactivity (figure 1); thus I-131-insulin could be separated from most serum proteins.

According to this elution pattern, the serum sample without addition of I-131-insulin was separated into two fractions; the first fraction which contained most of serum proteins was designated as Fraction A (Fr. A), and the second fraction, which corresponded to I-131-insulin in the pretested elution, was called Fraction B (Fr. B). Each of Fr. A or B was pooled, poured into a boiled Visking tubing (No. 18/32, Visking Co.) and placed in dry Sephadex G-25 powder (coarse) at 4° C. The content of the Visking tubing became concentrated without condensation of salts, since Sephadex powder absorbed both water and salts.[4] Wet Sephadex powder was replaced by dry powder each day. It took about five days to concentrate Fr. A (about 130 ml.) to 10 ml., and ten days to concentrate Fr. B (about 340 ml.) to the same volume.

These concentrated samples were then dialyzed at 4° C. in the same tubing against 1,000 ml. of Gey and Gey buffer (pH 7.4)[5] for forty-eight hours with a change of buffer after twenty-four hours. After dialysis, samples were frozen until assay. Over-all recoveries of I-131-insulin added to serum through gel filtration, concentration and dialysis ranged between 69 per cent and 82 per cent.

*Experiments with rat fat pad and the assay of ILA*

Male Wistar rats weighing 170-200 gm., fed ad libitum on Oriental rat chow (Oriental Kobo Kogyo Co., Tokyo), were decapitated, and the epididymal fat pads were removed and distributed according to balanced pool design.[6] Weights of tissues were calculated by weighing incubation flasks twice, before and after addition of tissues. Gey and Gey buffer containing 200 mg./100 ml. gelatin and 200 mg./100 ml. glucose was used for incubation. Samples were tested in duplicates at 1:10 dilution with this buffer after glucose concentration was adjusted to 200 mg./100 ml. Incubation was carried out in 2 ml. medium at 37.4° C. for two hours at 80 cycles/minutes either by Warburg manometric apparatus or by Dubnoff metabolic shaker. Capacities of flasks used in Warburg apparatus and in Dubnoff shaker were 15 ml. and 50 ml. respectively. Four different metabolic parameters were used for the assay of ILA. By Warburg apparatus, net gas exchange[7] and glucose uptake were measured. Glucose uptake was calculated from the difference of glucose concentrations in the medium before and after incubation. Glucose concentration was determined by glucose oxidase method using Glucostat (Worthington Biochemical Corporation, Freehold, N. J.). In other experiments using the Dubnoff shaker, 0.25 μc. D-glucose U-C-14 (specific activity 27-35 μc./mg., Daiichi Kagaku Yakuhin Co., Tokyo) was added to each flask, and C-14-$O_2$ production and C-14 incorporation into total lipid were measured as described previously.[8] Radioactivity was counted by a liquid scintillation counter (Ansitron Model-1300). In several experiments, in which C-14 incorporation into glycogen was measured, 1 μc. of D-glucose-U-C-14 was added to each flask, and the glucose concentration of the medium was reduced to 100 mg./100 ml. to increase the specific activity.

After incubating for two hours, fat pads were defatted twice with 20 ml. of chloroform-methanol (2:1 v/v) then heated in 2 ml. of 30 per cent KOH in boiled water bath. Glycogen was precipitated by 1.2 volumes of pure ethanol with 0.5 mg. nonlabeled glycogen as carrier, and allowed to stand overnight at 4° C. After centrifugation at 3,000 rpm for thirty minutes, the supernatant was decanted and the precipitate was dissolved in 0.5 ml. distilled water, then counted in Bray's naphthalene-dioxane-PPO-POPOP scintillator.[9]

Pork standard insulin (Novo, Lot No. S 1562, 23.5 U./mg., ten times recrystallized) was dissolved in 1/200 N HCl, stored frozen as 1 U./ml. and diluted to the desired concentration immediately before use. For the assay of ILA, 8, 31, 125 and 500 μU./ml. were used as standards.

*Anti-insulin serum and cysteine*

Sera obtained from several guinea pigs which received several injections of a mixture of pork insulin solution (Eli Lilly) and Freund complete adjuvant (Difco Lab.) were pooled. L-cysteine hydrochloride was purchased from Nippon Rikagaku Yakuhin Co., Tokyo.

*Localized intraperitoneal action on rat diaphragm and epididymal adipose tissue in vivo*

Male rats, weighing 140-160 gm., and fed ad libitum, were used for the study of intraperitoneal action according to the method of Rafaelsen et al.[10] On the day preceding the experiment, the testes were displaced into the abdominal cavity, and the communication between peritoneal and scrotal spaces was closed by a transcutaneous suture. On the experiment day, the animals received intraperitoneal injections of 2 μc. of glucose-U-C-14 with 2 ml. per rat of test samples. Each sample was injected to two rats. One hundred and fifty minutes after the injection, rats were decapitated and diaphragm and epididymal fat pads were excised. C-14 incorporation into total lipids and glycogen of the fat pads and C-14 incorporation into diaphragm glycogen were measured.

*Assay of immunoreactive insulin*

The immunoassay was carried out by a double antibody technic as described previously.[11] The optimal amount of rabbit anti-guinea pig globulin serum to ensure complete precipitation was carefully tested in the presence of each different fraction.[12] As the standard, crude dog insulin (3.0 U./mg.) was kindly prepared with its potency determined by a standard rabbit hypoglycemia method by Simizu Seiyaku Co.

## RESULTS

(1) *ILA and IRI values in serum, Fraction A and Fraction B*

The results of ILA and IRI measurements of serum and its fractions are shown in table 1. In the untreated sera, there was only a poor correlation between the ILA

TABLE 1

ILA, IRI and ILA/IRI ratio in various dog sera, and Fractions A and B separated by gel filtration with Sephadex G-50. ILA was assayed in 1:10 dilution by rat fat pad method using glucose uptake as index.

| Samples | Serum | | | Fraction A | | | Fraction B | | |
|---|---|---|---|---|---|---|---|---|---|
| | ILA (μU./ml.) | IRI (μU./ml.) | ILA/IRI | ILA (μU./ml.) | IRI (μU./ml.) | ILA/IRI | ILA (μU./ml.) | IRI (μU./ml.) | ILA/IRI |
| 1. Pancreatic vein serum at fasting | 4,200 | 1,115 | 3.8 | 1,260 | 7 | 180.0 | 1,310 | 853 | 1.5 |
| 2. Pancreatic vein serum at fasting | 3,600 | 485 | 7.4 | 1,550 | 10 | 155.0 | 460 | 303 | 1.5 |
| 3. Pancreatic vein serum at fasting | 1,425 | 90 | 17.8 | 1,400 | 13 | 107.7 | < 310 | 73 | — |
| 4. Pancreatic vein serum at fasting | 2,550 | 1,690 | 1.5 | 1,250 | 14 | 89.3 | 1,500 | 690 | 2.2 |
| 5. Pancreatic vein serum after glucose injection | 2,875 | 2,070 | 1.4 | 1,750 | 8 | 218.8 | 3,475 | 1,780 | 2.0 |
| 6. Pancreatic vein serum after glucose injection | 3,450 | 2,310 | 1.5 | 1,650 | 14 | 117.9 | 1,330 | 1,260 | 1.1 |
| 7. Femoral vein serum at fasting | 700 | 31 | 22.6 | 1,330 | 8 | 166.2 | 138 | 16 | 8.6 |
| 8. Femoral vein serum at fasting | 1,200 | 33 | 36.4 | 1,600 | 12 | 133.5 | 350 | 20 | 17.5 |
| 9. Femoral vein serum after glucose injection | 1,300 | 127 | 10.3 | 1,740 | 11 | 158.2 | 560 | 113 | 5.0 |
| 10. Diabetic dog serum at fasting | 430 | < 2 | — | 1,450 | < 2 | — | < 80 | < 2 | — |

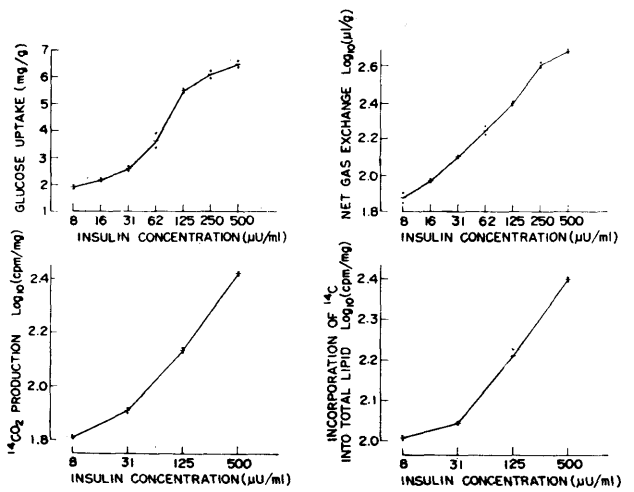


FIG. 2. Four different insulin dose-response curves by different parameters of fat pad metabolism. Parameters are glucose uptake, net gas exchange, C-14-$O_2$ production from D-glucose-U-C-14, and incorporation of C-14 into total lipid, respectively. The shapes of these four curves were relatively similar to each other over the same range of insulin doses.

and IRI values, and the ILA/IRI ratio scattered between 1.4 (pancreatic vein serum after glucose injection) and 36.4 (femoral vein serum at fasting). ILA values were always higher than the IRI.

After gel filtration, about 77 per cent of IRI in the original serum was recovered in Fr. B, whereas less than 11 per cent of original IRI was detected in Fr. A.

On the other hand, recoveries of ILA in fractions were very variable. Fr. B had relatively similar ILA values to corresponding IRI for the pancreatic vein sera. ILA/IRI ratios in Fr. B of the peripheral vein sera were fairly high, but still much less than those of the original sera. Considerable ILA was demonstrated in Fr. A, although there was very little IRI in this fraction. ILA values of this fraction were fairly constant, ranging between 1,250 and 1,750 μU./ml., irrespectively of original serum whether it was taken from the pancreatic vein or the peripheral vein and whether at fasting or after glucose injection. In some instances, ILA of Fr. A exceeded that of the original serum.

A serum from a dog, which had been depancreatized two days before, showed the ILA of 430 μU./ml. with IRI less than 2 μU./ml. the minimal detectable range in our present immunoassay. Fr. A from this serum also revealed the ILA of the same degree as other sera.

(2) *ILA values of serum and fractions based on various parameters of fat pad metabolism*

Figure 2 shows four different dose-response curves with standard insulin, each of which is based on glucose uptake, net gas exchange, C-14 incorporation into $CO_2$ or total lipid respectively. The ranges of linearity were fairly similar between these curves. As shown in table 2, the fat pad assay based on each of these four different parameters gave comparable values of ILA for both the untreated pancreatic vein serum and fractions.

TABLE 2

ILA values of dog pancreatic vein sera, Fractions A and B assayed by four different parameters of fat pad metabolism. Comparable values of ILA were obtained by each of these parameters (i.e., glucose uptake, net gas exchange, C-14 incorporation from D-glucose-U-C-14 into $CO_2$ or total lipid).

| Samples | Parameters: Glucose uptake (μU./ml.) | Net gas exchange (μU./ml.) | C-14-$O_2$ production (μU./ml.) | C-14 incorporation into total lipid (μU./ml.) | IRI (μU./ml.) |
|---|---|---|---|---|---|
| Serum at fasting | 4,200 | 4,800 | 4,300 | 4,600 | 1,115 |
| 1. Fraction A | 1,260 | 1,300 | 1,250 | 1,580 | 7 |
| Fraction B | 1,310 | 1,550 | 1,250 | 1,680 | 853 |
| Serum at fasting | 3,600 | 3,400 | 3,350 | 3,000 | 485 |
| 2. Fraction A | 1,550 | 1,870 | 1,105 | 875 | 10 |
| Fraction B | 460 | 385 | 425 | 400 | 303 |
| Serum at fasting | 2,550 | 1,900 | 1,980 | 2,780 | 1,690 |
| 3. Fraction A | 1,250 | 920 | 1,200 | 1,370 | 14 |
| Fraction B | 1,500 | 1,300 | 860 | 1,320 | 690 |
| Serum after glucose injection | 3,450 | 3,250 | 3,000 | 2,500 | 2,310 |
| 4. Fraction A | 1,650 | 1,650 | 1,900 | 1,550 | 14 |
| Fraction B | 1,330 | 1,540 | 1,870 | 1,300 | 1,260 |

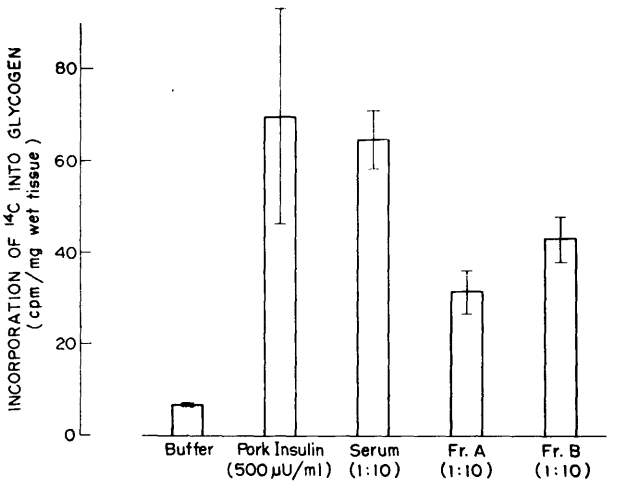


FIG. 3. Incorporation of C-14 into glycogen of rat epididymal adipose tissue from D-glucose-U-C-14 in vitro. Each bar represents the mean and standard error of three experiments. Insulin, Fractions A and B all stimulated C-14 incorporation into fat pad glycogen.

(3) *Effect of serum and fractions on glycogen synthesis by fat pad*

Crystalline insulin, untreated serum, Fr. A and Fr. B, all increased the incorporation of C-14 into glycogen from D-glucose-U-C-14 (figure 3).

(4) *Effect of intraperitoneal injection of serum or fractions*

C-14 incorporations from glucose-U-C-14 into glycogen of diaphragm and total lipid of fat pads were stimulated by intraperitoneal injection of each of insulin, serum and fractions.

C-14 incorporation into fat pad glycogen was stimulated by 10,000 μU./ml. insulin but less clearly by 1,000 μU./ml. insulin, serum or fractions (table 3).

(5) *Inhibition of ILA by anti-insulin serum and cysteine*

Thirty minutes prior to addition of tissue pieces, 0.1 ml. guinea pig anti-insulin serum was added to each flask containing 2 ml. incubation medium. This amount of antiserum completely neutralized 2,000 μU. of pork insulin. The same amount of serum from normal guinea pig was added to control flasks. By addition of antiserum, the ILA of untreated serum and of Fr. B were suppressed partially and considerably, but no suppression was noted for the ILA of Fr. A (figure 4, above). In another experiment, L-cysteine hydrochloride was added to each sample in a final concentration of 0.02 M and incubated for ninety minutes under $N_2$ gas, then dialyzed at 4° C. overnight against Gey and Gey buffer containing glucose and gelatin. This treatment suppressed the actions of pork insulin and Fr. B nearly to the basal level. Inhibition of the ILA of serum was partial, and the ILA of Fr. A was decreased only slightly (figure 4, below).

(6) *Test of parallelism between regression lines of insulin and serum or fractions*

By the use of $CO_2$-14 production, net gas exchange and glucose uptake as indexes of ILA, 2 × 2 point test was performed to compare the slopes of the regression lines of standard insulin and of serum or fractions. Samples were selected in order to bring both the high dose and the low dose within the linear range of dose-response curves. Deviation from parallelism was significant between insulin and a serum with IRI of 226 μU./ml., and between insulin and Fr. A. No significant deviation from parallelism was demonstrated, however, between insulin and a serum with IRI of 1,115 μU./ml., and between insulin and Fr. B (figure 5).

To test further the deviation from parallelism of dose-response curves of Fr. A samples and of insulin, five additional Fr. A samples were tested in four to five dilutions (figure 6). Deviation was significant in all of these five experiments.

(7) *Effect of dilution-reconcentration and dialysis of serum on ILA*

TABLE 3

C-14 incorporation from glucose-U-C-14 into glycogen of diaphragm and total lipid of fat pads and into fat pad glycogen. 2 μc. of glucose-U-C-14 was administered intraperitoneally with insulin, serum or fractions. Each value is the mean of a duplicate, expressed in cpm/mg. wet tissue weight.

| Samples | Diaphragm glycogen | | Total lipid of fat pad | | Fat pad glycogen | |
|---|---|---|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 |
| Buffer | 5,813 | 3,570 | 12,790 | 8,095 | 740 | 816 |
| Insulin 1,000 μU./ml. | 28,675 | 8,620 | 57,500 | 28,400 | 3,690 | 446 |
| Insulin 10,000 μU./ml. | 83,300 | 23,310 | 128,550 | 60,925 | 16,535 | 11,865 |
| Serum | 45,950 | 27,625 | 94,450 | 56,450 | 5,695 | 2,390 |
| Fr. A | 10,613 | 6,470 | 49,575 | 33,743 | 517 | 867 |
| Fr. B | 21,845 | 8,860 | 77,660 | 23,875 | 1,204 | 1,831 |

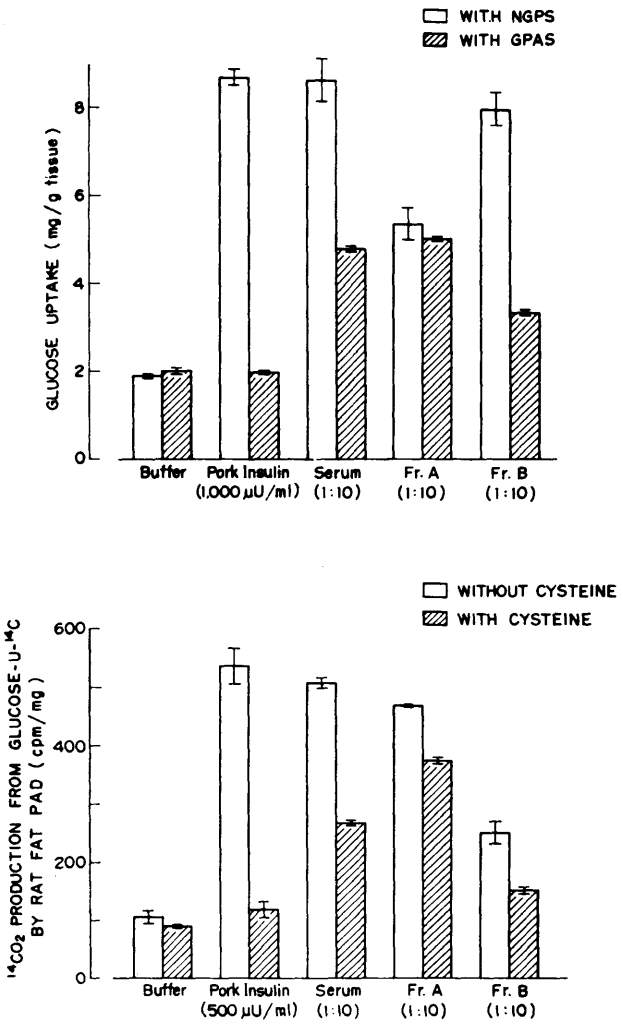


FIG. 4. Suppression of effects of insulin, serum, Fractions A and B by anti-insulin serum or by cysteine treatment. Each bar represents the mean of duplicate estimations. Above: Normal guinea pig serum (NGPS) was added to control flasks. Addition of guinea pig anti-insulin serum (GPAS) completely suppressed the effect of insulin and mostly that of Fr. B, while the effect of Fr. A was not affected by anti-serum and that of serum was partially inhibited. Below: Effects of insulin and of Fr. B were suppressed by cysteine near the basal level, while those of serum and Fr. A were partially suppressed.

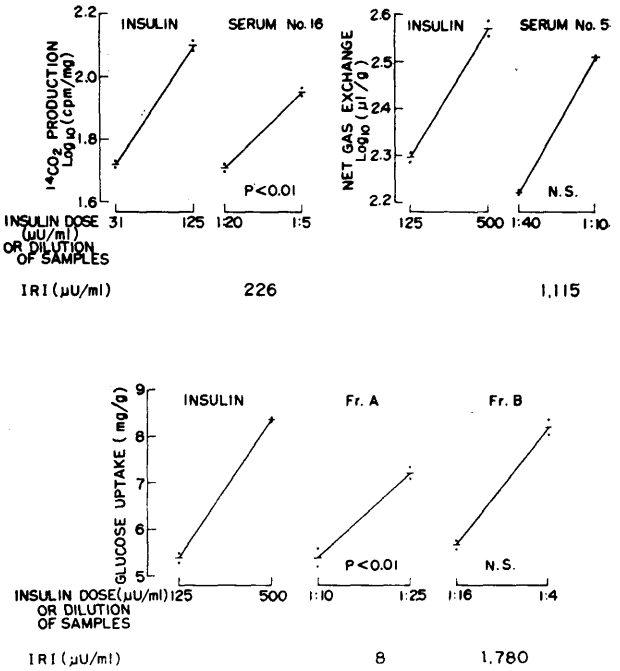


FIG. 5. Test of parallelism between standard insulin and serum or fractions with fat pad assay. Response of fat pad is expressed by C-14-$O_2$ production from D-glucose-U-C-14 in $\log_{10}$ (cpm/mg.), net gas exchange in $\log_{10}$ ($\mu$l./gm.), and glucose uptake in mg./gm., respectively, as shown in the figure. When these parameters were measured simultaneously in the same experiments, the ultimate conclusions for parallelism were the same irrespective of different parameters.

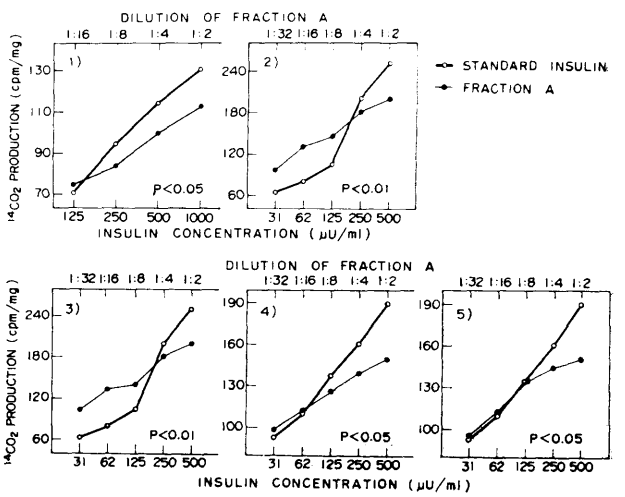


FIG. 6 (at right). Test of parallelism between standard insulin and Fr. A. samples. Deviation from parallelism was significant in all of five samples:
(1) Fr. A from pancreatic vein serum after glucose injection.
(2) Fr. A from pancreatic vein serum at fasting.
(3) Fr. A from pancreatic vein serum after glucose injection.
(4) Fr. A from femoral vein serum at fasting.
(5) Fr. A from femoral vein serum after glucose injection.

Ten milliliters of serum were diluted to 500 ml. with Tris-HCl buffer, poured in Visking tubing and concentrated to 10 ml. in dry Sephadex G-25 powder, then dialyzed against 1,000 ml. Gey and Gey buffer at 4° C. for forty-eight hours. This treatment resulted in marked increase in ILA for each serum. Simple dialysis of other sera in a similar manner did not cause any significant increase in their ILA (table 4).

(8) *Acid-ethanol-NaCl extraction of Fr. A*

Several samples of Fr. A from peripheral vein sera were extracted with acid-ethanol-NaCl as described previously.[2] ILA of extract of Fr. A was not detectable in 1:10 dilution, nor there was any indication of increases in ILA or suppressible ILA after extraction (table 5).

TABLE 4

Comparison of ILA in the untreated serum, dialyzed serum and diluted-reconcentrated serum. Sera were diluted, concentrated and dialyzed or simply dialyzed and their ILA values were measured by the use of C-14-$O_2$ production. Dilution followed by reconcentration and dialysis caused marked increase in ILA, but simple dialysis did not increase ILA values.

| Samples | Original serum (μU./ml.) | Diluted, concentrated and dialyzed (μU./ml.) | Dialyzed (μU./ml.) |
|---|---|---|---|
| Pancreatic vein serum after glucose injection | 1,420 | 2,650 | — |
| Diabetic dog serum | 460 | 950 | — |
| Femoral vein serum at fasting | 480 | — | 590 |
| Pancreatic vein serum after glucose injection | 3,100 | — | 2,900 |
| Diabetic dog serum | 610 | — | 545 |

## DISCUSSION

By gel filtration with Sephadex G-50, IRI in serum was recovered consistently in Fr. B, separated from most serum proteins which were eluted in Fr. A. In this elution pattern, serum IRI behaved similarly to I-131-insulin added to serum. The average over-all recovery of IRI after gel filtration, concentration and dialysis was about 88 per cent (11 per cent in Fr. A and 77 per cent in Fr. B) and always less than 100 per cent. On the other hand, ILA by fat pad method was demonstrated both in Fr. A and Fr. B. This distribution pattern of ILA and IRI is similar to results of Steinke and Soeldner[13] in that the protein fraction contains considerably high ILA but very little IRI. In our experiments, however, the sum of the IRI after fractionation never exceeded that of IRI in the original serum. By means of gel filtration, Poffenbarger et al. estimated that the ILA in human serum has a molecular weight of about 40,000-50,000.[14]

The ILA in each fraction shared many common features with standard insulin in its stimulating effects on the adipose tissue metabolism in vitro and on the diaphragm and fat pads in vivo by intraperitoneal injection. ILA values for each of serum, Fr. A and Fr. B obtained by four different metabolic parameters were fairly similar to each other.

The ILA in Fr. B corresponded better to IRI (i.e., ILA/IRI ratios being closer to 1.0) than the ILA in the untreated serum. Besides its stimulating effects on fat pad metabolism, this ILA was suppressed both by

TABLE 5

Effect of anti-insulin serum on ILA of Fraction A and its salt-ethanol extracts. ILA of Fraction A was not extracted, and both Fraction A and its extracts were not suppressed by anti-insulin serum. All values are in cpm. mg. wet tissue weight. Difference between treatments were tested by *t* test.

| Incubation medium | | No. of experiments | Mean ± S.D. (cpm/mg.) | Significance |
|---|---|---|---|---|
| a. Buffer | + NGPS | 5 | 89.4± 15.3 | |
| b. Buffer | + GPAS | 5 | 79.5± 8.7 | |
| c. Insulin 500 μU./ml. | + NGPS | 5 | 273.9± 53.7 | a-b, e-f, g-h, b-d, b-h: not significant |
| d. Insulin 500 μU./ml. | + GPAS | 5 | 75.4± 18.7 | |
| e. Fraction A (1:10) | + NGPS | 3 | 303.8±155.9 | |
| f. Fraction A (1:10) | + GPAS | 3 | 309.5±171.3 | |
| g. Salt-ethanol extract of Fraction A (1:10) | + NGPS | 3 | 92.4± 1.6 | c-d, b-f: $p < 0.01$ |
| h. Salt-ethanol extract of Fraction A (1:10) | + GPAS | 3 | 85.3± 3.8 | |

anti-insulin serum and by cysteine, and its dose-response line was parallel to that of standard insulin. All these data suggest that the ILA in Fr. B is primarily due to "insulin" in the blood which has the same immunological and biological activities as the pancreatic insulin. In some samples with low IRI values, however, ILA values in Fr. B were considerably higher than the corresponding IRI. This discrepancy might be attributed, at least in part, to contamination by ILA in Fr. A, or there might be some factors of fairly low molecular weight with nonimmunoreactive insulin-like activity in Fr. B, such as demonstrated in "purified extract B" of Bürgi et al.[15,16]

Although the stimulatory effects of Fr. A on rat fat pad were also similar to insulin in many aspects including C-14 incorporation into glycogen, it showed several different properties from that of insulin. This ILA was only slightly suppressed with cysteine, and not neutralized by anti-insulin serum corresponding to the very low levels of insulin measurable by immunoassay. Its regression line was not parallel to that of insulin. A serum from a depancreatized dog had an appreciable ILA in this fraction despite the absence of measurable IRI. As no satisfactory method of identification of minute insulin in blood is available at present, it seems better to consider serum ILA as a separate factor from insulin unless it fulfills all the known properties of authentic insulin. From this point of view, the ILA of Fr. A cannot be called insulin. The lack of parallelism between the slopes of regression line of ILA in Fr. A and that of insulin is equivalent to so-called "dilution effect" and indicates that this ILA cannot be adequately expressed as "microunits" of crystalline insulin.

The ILA in Fr. A appears to resemble "bound insulin" (Antoniades[17,18]) in its elution pattern by gel filtration and nonsuppressibility by anti-insulin serum, but this ILA did not change before and after glucose loading whereas "bound insulin" decreases after glucose. It appears also similar to "nonsuppressible ILA" (Froesch et al.[6]) or "atypical insulin" (Samaan[19]). The invariability of this ILA by glucose loading resembles the "nonsuppressible ILA." The ILA which lacks immunoreactivity with anti-insulin serum was extracted only to a little extent from human or dog serum. Bürgi et al. reported a successful extraction of nonsuppressible ILA from a Cohn fraction of human plasma by acid-ethanol.[15] However, their recovery of nonsuppressible ILA was low, and the active material obtained in "extract B" differed much in its molecular weight from that in the native serum.[15,16] Unlike Samaan,[19] no evidence was obtained in our hands to suggest a conversion of nonimmunoreactive ILA to immunoreactive ILA. The ILA of Fr. A was fairly constant regardless of the origin of sera. When the femoral vein sera of normal or depancreatized dogs were fractionated, higher ILA than the original sera were sometimes observed in Fr. A. This ILA appears to be affected by relatively slight change of physicochemical state of serum proteins, because it was demonstrated that a simple dilution of the normal or diabetic dog serum with buffer followed by a subsequent concentration in dry Sephadex powder was accompanied by an increase in ILA. Similar observations were also made by Rasio et al.[20] It seems unlikely that this increase in activity is caused by removal of antagonist through the wall of Visking tubing, because simple dialysis of serum against Gey and Gey buffer does not cause such a marked increase in ILA. Such increases of ILA of serum or its fractions by treatments have been reported also by Lyngsøe[21] with tap water dialysis, and by Power et al.[22] with storage of serum in frozen state.

The similarity of ILA in Fr. A of the peripheral vein and the pancreatic vein sera and its persistence after pancreatectomy suggest its nonpancreatic origin. The apparent in vivo ineffectiveness of this ILA in the depancreatized dogs and relative constancy in different physiologic conditions throw some doubts on its physiological role as a regulator of metabolism. As only very little ILA was detectable in the lymph, Cahill et al.[23] expressed their doubt on the capillary permeability of serum ILA. Solomon et al.[24] reported that porcine nonsuppressible insulin-like activity has an action similar to insulin on myocardium of isolated perfused rat heart through "normal" vascular tree. Although ILA in Fr. A seems to be an interesting factor, its significance in the body still seems to be a riddle at present.

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