# Inhibitory Effects of "Bound" Insulin on Insulin Uptake by Isolated Tissues

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SUMMARY

"Bound" insulin preparations obtained from pooled human sera inhibited the uptake of crystalline insulin by isolated rat muscle (hemidiaphragm) and epididymal adipose tissue. When crystalline insulin alone was incubated with isolated tissues, its concentration in the incubating medium declined gradually, as shown by immunoassay of samples obtained at various intervals during the incubation. Addition of "bound" insulin preparations into the incubation medium greatly reduced the rate of disappearance of the crystalline insulin, "bound" insulin inhibiting the uptake of insulin by the tissues. The inhibition of insulin uptake by muscle, caused by "bound" insulin, was accompanied by inhibition of the biologic activity of insulin on this tissue.

Preincubation of the isolated tissues with "bound" insulin, with whole fasting human sera from maturity-onset diabetics, or with crystalline insulin, prior to the addition of crystalline insulin, also resulted in significant inhibition of insulin uptake by these tissues.

Synalbumin preparations obtained from fasting human sera by acid-ethanol extraction, as described by Vallance-Owen and collaborators, also inhibited the uptake of crystalline insulin by isolated muscle and adipose tissue and the biologic activity of insulin on muscle. Like "bound" insulin, the synalbumin extracts were inactive on isolated muscle and unreactive with anti-insulin antisera but exerted insulin-like activity on isolated adipose tissue. Their action when injected intraperitoneally into intact rats was similar to that of "bound" and crystalline insulin on the muscle and the adipose tissue. It is suggested that the synalbumin preparations obtained from sera by acid-ethanol extraction may contain the bulk of the serum "bound" insulin which contributes, at least in part, to the insulin inhibitory properties of these preparations. DIABETES *15*:655-62, September, 1966.

Studies from this laboratory have suggested the possibility that "free" insulin released by the pancreas may be metabolized in vivo by extrapancreatic tissues into a form which we termed "bound" insulin.[1] "Bound" insulin exhibits a higher molecular weight and a slower electrophoretic mobility than "free" insulin and is unreactive with anti-insulin antisera.[2] We suggested that "bound" insulin represents a combination of "free" insulin with other macromolecules, or a combination of "free" insulin molecules, such a combination being catalyzed in vivo by extrapancreatic tissues. It is also possible that "bound" insulin represents a modified form of "free" insulin, this modified insulin being attached to macromolecules.[2]

"Bound" insulin preparations injected intravenously or intraperitoneally into rats or mice exhibited biologic effects similar to those of crystalline insulin.[3-5] In vitro "bound" insulin exerted biologic activity on isolated rat adipose tissue but not on isolated muscle, unless adipose tissue extract (ATE) was added into the incubation medium.[6-8]

Recently we reported that "bound" insulin preparations could inhibit the uptake of crystalline insulin by isolated muscle. When crystalline insulin was incubated with isolated rat diaphragm, the insulin rapidly disappeared from the incubation medium, as shown by immunoassay of samples collected from the incubate at various intervals. Addition of "bound" insulin into the incubation medium, or preincubation of the tissues with "bound" insulin prior to the addition of crystalline insulin, produced a significant reduction in the rate of disappearance of insulin from the incubation medium.[9]

In this report studies are presented which demonstrate that "bound" insulin inhibits the normal uptake of crystalline insulin by both isolated muscle and adipose tissue. Preincubation of isolated muscle or adipose tissue with "bound" or crystalline insulin or whole undiluted serum, prior to the addition of crystalline insulin, similarly produced significant inhibition of insulin uptake by these tissues. The inhibition of crystalline insulin uptake by muscle, caused by "bound" insulin, was accompanied by a significant inhibition of the biologic activity of crystalline insulin.

Studies on the synalbumin preparations of Vallance-Owen and collaborators[10-13] suggest the possibility that these preparations, which antagonize insulin activity on

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isolated muscle, may contain, among other substances, a large portion of the serum "bound" insulin.

## MATERIAL AND METHODS

Partially purified "bound" insulin preparations were obtained from pooled normal human sera by resin adsorption and elution as described elsewhere.[2] The specific activity of the partially purified preparations of "bound" insulin used in these studies was about 900 microunits per mg. protein as measured on isolated rat hemidiaphragm in the presence of adipose tissue extracts (ATE).[6] One milligram of the partially purified "bound" insulin preparation exhibited less than 10 microunits of insulin by the Morgan and Lazarow immunoassay technic.[15]

Synalbumin preparations from fasting sera were obtained by acid-ethanol extraction of the serum proteins by the method of Debro et al.[14] as modified by Vallance-Owen et al.[10] The sera were mixed with equal volumes of 10 per cent trichloroacetic acid (TCA). The supernatant fluid was discarded and the precipitate was washed with one serum volumes of 5 per cent TCA. The precipitate was then extracted with three serum volumes of 1 per cent TCA in 95 per cent EtOH (*p*H 2.1). After centrifugation, the extract was dialyzed against running cold water for forty-eight hours, lyopholized, reconstituted to the original serum volume, the *p*H adjusted to 7.4 and dialyzed against 100 volumes of Gey and Gey bicarbonate buffer for forty-eight hours at 2° C. with four changes. The insulin content of the reconstituted synalbumin preparations was less than 10 microunits per milliliter as measured by the Morgan and Lazarow immunoassay technic.[15]

All samples containing crystalline or "bound" insulin were made in 3 per cent Human Plasma Albumin (HPA) (Protein Foundation reworked Fraction V). The albumin and "bound" insulin solutions were dialyzed before use for forty-eight hours at 2° C., with two changes, against 100 volumes of Gey and Gey bicarbonate buffer. Sera collected from fasting untreated maturity-onset diabetics were similarly dialyzed against Gey and Gey bicarbonate buffer.

Isolated rat hemidiaphragms or epididymal adipose tissues were obtained from Charles River Laboratories, male, CD rats weighing 106 to 114 gm. Wet hemidiaphragms used weighed between 90 to 100 mg. and isolated epididymal adipose tissue pad, 180 to 220 mg.

Two milliliters of crystalline insulin alone (400 μU./ml. in 3 per cent HPA), or crystalline insulin with "bound" insulin (1 mg./ml. in 3 per cent HPA) were incubated with each hemidiaphragm or epididymal adipose tissue for ninety minutes at 37° C. Samples (0.1 ml.) of media were obtained at 10, 20, 40, 60 and 90 min. during the incubation and immunoassayed by the Morgan and Lazarow technic.[15] Before incubation the samples were equilibrated for five minutes with 95 per cent $O_2$:5 per cent $CO_2$.

In other studies hemidiaphragms or adipose tissues were incubated in 2 ml. of "bound" (1 mg./ml.) or crystalline insulin (800 μU./ml.), in 3 per cent HPA, or in 4 ml. of undiluted serum or in 4 ml. of synalbumin solution, for forty minutes at 37° C., after which the incubate was discarded. The tissues were then incubated with 2 ml. of crystalline insulin media (400 μU./ml. in 3 per cent HPA) for ninety minutes at 37° C. Samples were obtained during the incubation as described above and immunoassayed. Before each incubation all samples were equilibrated with 95 per cent $O_2$:5 per cent $CO_2$.

Biologic activity of insulin on isolated hemidiaphragm from fasted (24-hr.) rats was measured by the technic of Vallance-Owen and Hurlock.[16] The glucose content in all incubating systems was 2.8 mg. per ml. The uptake of glucose by the isolated muscle was used as the measure of insulin activity.

The insulin activity on isolated rat epididymal adipose tissue was measured by the technic described by Ball et al.[17] Pieces of isolated epididymal adipose tissue from fed rats were incubated with the samples in Warburg flasks at 37° C. in Gey and Gey bicarbonate buffer, equilibrated with 95 per cent $O_2$:5 per cent $CO_2$, containing 3 mg. of glucose per milliliter. Net gas exchange was measured for three hours and expressed as μl $CO_2$ per 100 mg. wet weight adipose tissue.

In vivo insulin activity was measured by the intraperitoneal technic described by Rafaelsen et al.[18] Five milliliters of 3 per cent HPA containing a trace of glucose-u-C-14 with or without synalbumin preparations, "bound" insulin or crystalline insulin were injected intraperitoneally into groups of eight, intact, fed rats. Two hours after the injection, the rats were narcotized lightly with 50 per cent $CO_2$:50 per cent $O_2$, decapitated, and the two hemidiaphragms and epididymal adipose tissues were rapidly removed, weighed and processed for the extraction of glycogen or fat as described elsewhere.[18] The radioactivity in the glycogen of the muscle or adipose tissue, or the fat of the adipose tissue was counted and expressed as CPM per gm. wet tissue.

## RESULTS

Incubation of crystalline insulin with isolated rat

muscle resulted in the rapid disappearance of insulin from the incubation medium as shown by immunoassay of samples of the medium obtained at various intervals during the incubation (figure 1). When crystalline insulin was incubated with isolated rat epididymal adipose tissue, its rate of disappearance from the incubation medium was slower. Addition of "bound" insulin concentrate (1 mg./ml.) into the incubation medium containing the crystalline insulin resulted in significant inhibition of insulin uptake by both isolated muscle and adipose tissue (figure 1).

The rate of disappearance of insulin was greater in the presence of isolated muscle from fed rats than from fasted rats. On the other hand, insulin disappeared more rapidly in the presence of isolated adipose tissue from fasted than fed rats.

The inhibition of insulin uptake by muscle, caused by "bound" insulin, was accompanied by a significant inhibition of the biologic activity of crystalline insulin (table 1). "Bound" insulin itself was biologically inactive on isolated muscle.

Preincubation of isolated muscle or adipose tissue with crystalline or "bound" insulin resulted in the inhibition of insulin uptake by these tissues (figure 2).

TABLE 1

Inhibition of biologic activity of crystalline insulin on isolated rat hemidiaphragm by "bound" insulin

| Samples | Glucose uptake ($\mu$g. glucose per 10 mg. dry diaphragm) | |
|---|---|---|
| Buffer alone | 187±14 | |
| "Bound" insulin alone (1 mg. protein/ml.) | 201±8 | |
| Crystalline insulin alone (500 $\mu$U./ml.) | 326±17 | p<.001 |
| "Bound" insulin (1 mg./ml.) plus crystalline insulin (500 $\mu$U./ml.) | 216±15 | |

All samples contained 30 mg. human plasma albumin per milliliter and were incubated with hemidiaphragms from fasted (24-hr.) rats. Values are means of eight incubations ± S.E.M.

Similar results were obtained when tissues were first preincubated with whole, undiluted sera from untreated diabetics (figure 3) or with synalbumin preparations obtained from normal sera (figure 4). Synalbumin, like "bound" insulin, also inhibited biologic activity of crystalline insulin on isolated rat hemidiaphragm (table 2), confirming the claim of Vallance-Owen et al.[10]

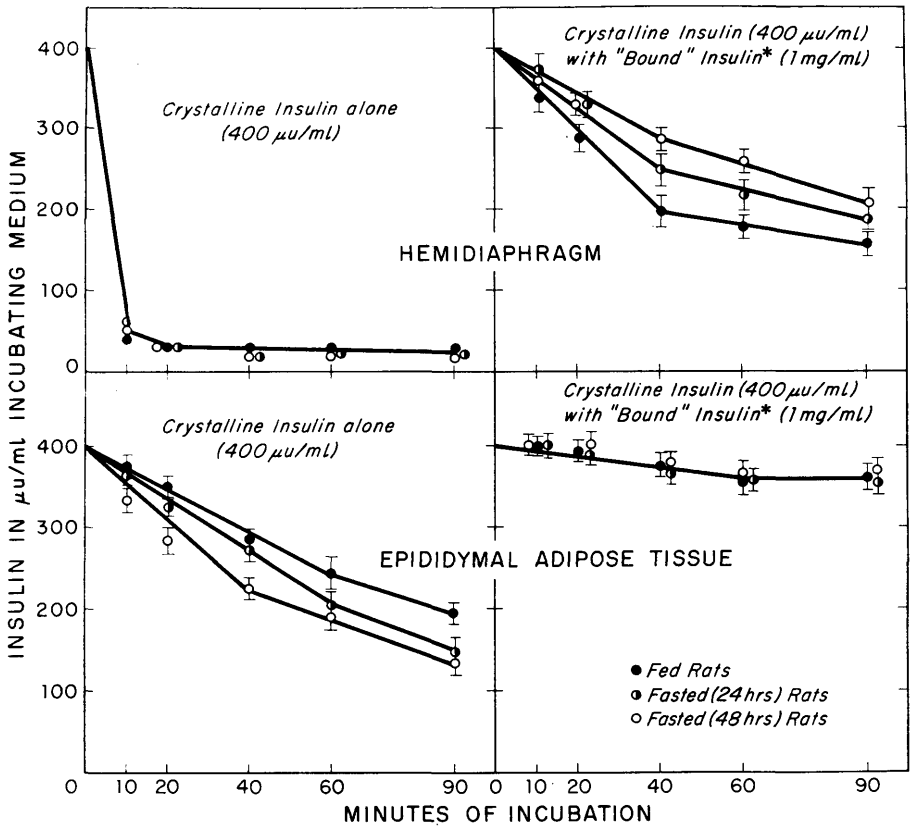


FIGURE 1.

Inhibitory effect of "bound" insulin on the uptake of crystalline insulin by isolated hemidiaphragm or adipose tissue as measured by immunoassay.

All incubations were carried out in the presence of 3 per cent human plasma albumin. Each tissue was incubated with 2 ml. of sample. Values represent the mean of six incubations ± S.E.M.

*One milligram protein of the partially purified serum "bound" insulin used assayed 900 $\mu$U. insulin activity on isolated rat hemidiaphragm in the presence of adipose tissue extracts (ATE).

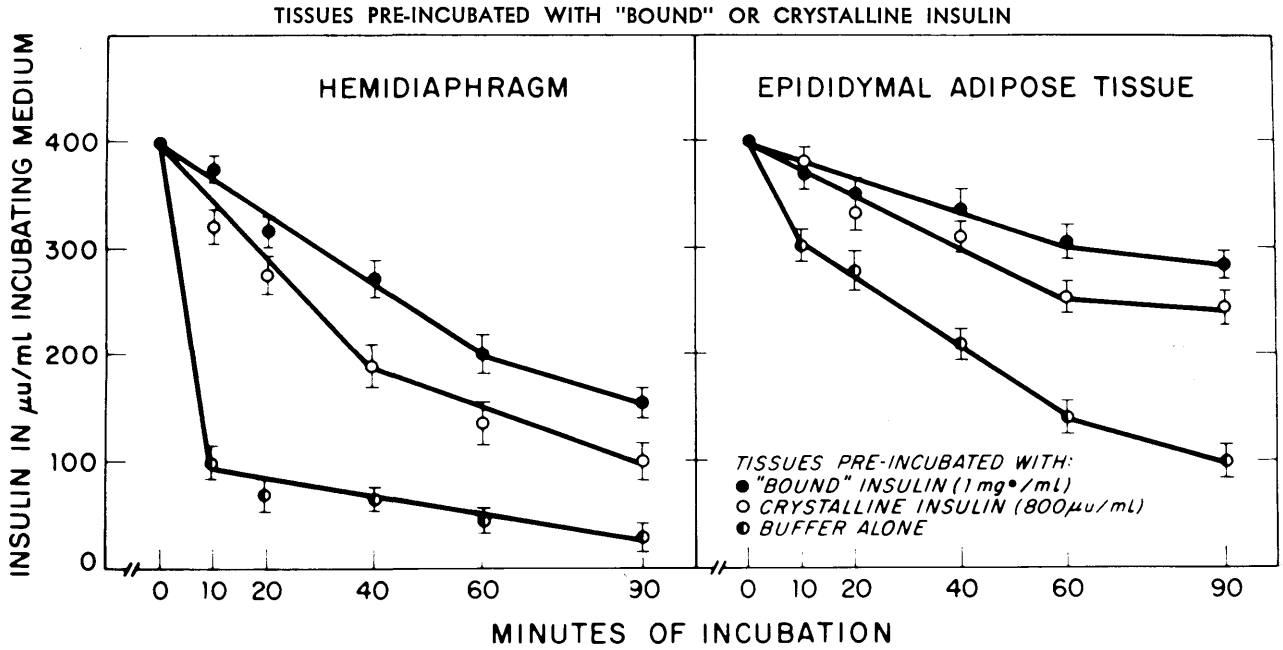


FIG. 2. Inhibition of crystalline insulin uptake by isolated tissues following preincubation of the tissues with "bound" or crystalline insulin.

All incubations were carried out in the presence of 3 per cent human plasma albumin (HPA). Each tissue was preincubated for 40 min. with 2 ml. of buffer, in 3 per cent HPA, without or with "bound" or crystalline insulin. The incubate was discarded, 2 ml. of buffer in 3 per cent HPA containing 400 μU./ml. crystalline insulin were added to each tissue and samples collected during the incubation and immunoassayed for insulin. Values represent the mean of six incubations ± S.E.M.

One mg. protein of the partially purified serum "bound" insulin used assayed 900 μU. insulin activity on isolated rat hemidiaphragm in the presence of adipose tissue extracts (ATE).

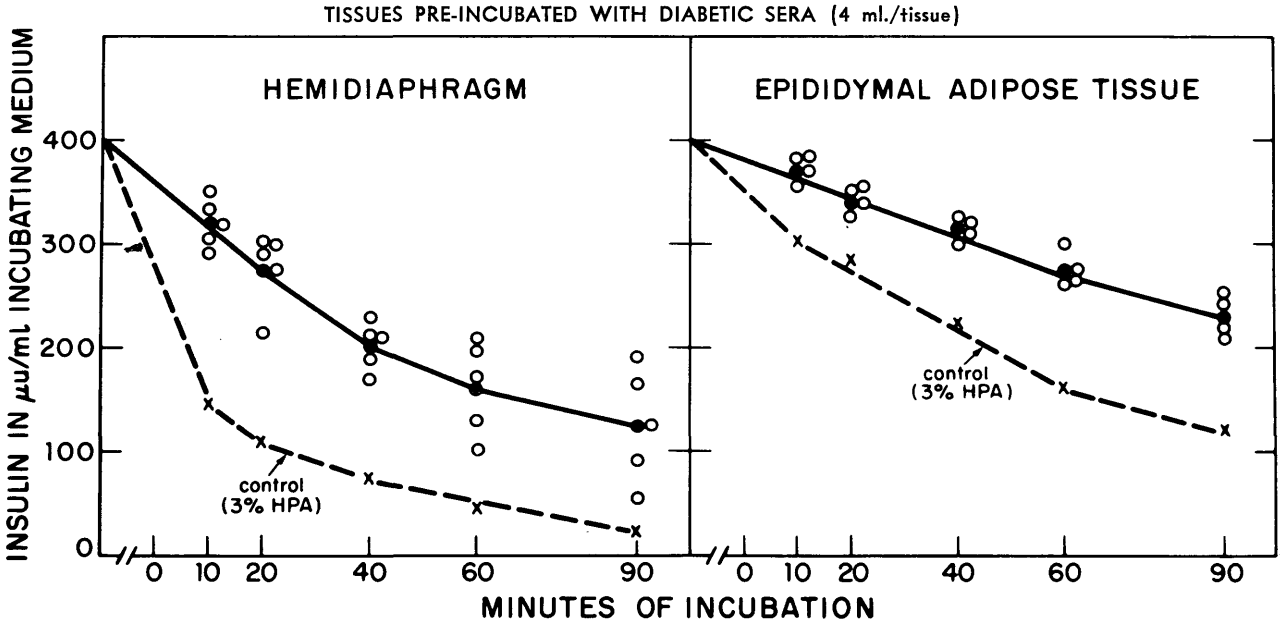


FIG. 3. Inhibition of crystalline insulin uptake by isolated tissues following preincubation of the tissues with whole, undiluted sera from newly discovered, noninsulin dependent diabetics.

Duplicate samples from each serum, or control buffer in 3 per cent HPA, were incubated with muscle and adipose tissue at 37°C. for 40 min. after which the incubate was discarded. Two milliliters of crystalline insulin (400 μU./ml.) in 3 per cent HPA were added to each preincubated tissue, and the incubation was continued for 90 min. Samples were collected during the incubation and immunoassayed for insulin. The mean values of five sera are presented by the dark circles.

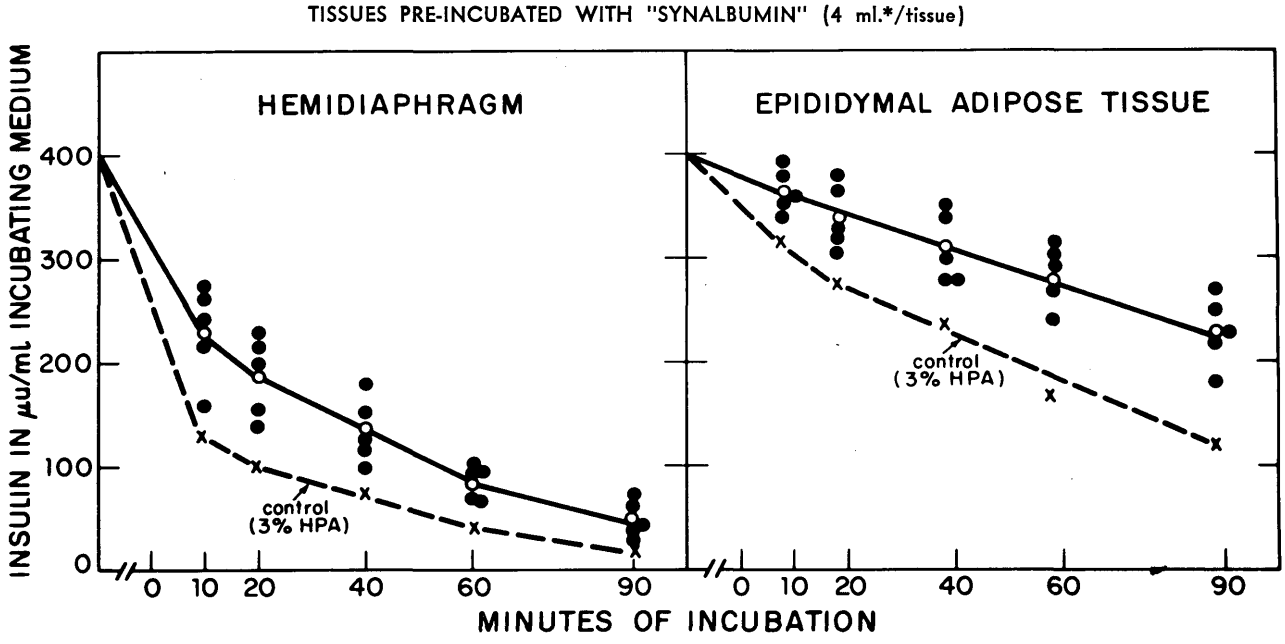


FIG. 4. Inhibition of crystalline insulin uptake by isolated tissues following preincubation of the tissue with synalbumin preparations obtained from normal fasting sera.

Duplicate samples from each synalbumin preparation, or control buffer in 3 per cent HPA, were incubated with muscle and adipose tissue at 37° C. for 40 min., after which the incubate was discarded. Two milliliters of crystalline insulin (400 μU./ml.) in 3 per cent HPA were added to each preincubated tissue, and the incubation was continued for 90 min. Samples were collected during the incubation and immunoassayed for insulin. The mean values of five synalbumin preparations are presented by the open circles.

TABLE 2

Inhibition of the activity of crystalline insulin on isolated muscle by "synalbumin"

| Samples | Glucose uptake (μg. glucose per 10 mg. dry diaphragm) | |
|---|---|---|
| Buffer alone | 176±8 | |
| "Synalbumin" alone (50 mg. protein/ml.) | 192±17 | |
| Crystalline insulin alone (500 μU./ml.) | 307±16 | p<.001 |
| "Synalbumin" (50 mg./ml.) plus crystalline insulin (500 μU./ml.) | 205±18 | |

The buffer and the crystalline insulin solution contained 30 mg. human plasma albumin per milliliter. All samples were incubated with hemidiaphragms obtained from fasted (24 hr.) rats. Values are means of eight incubations ± S.E.M.

In order to find out whether the inhibition of insulin uptake by isolated tissues caused by synalbumin preparations was due, at least in part, to the presence of "bound" insulin in these extracts, synalbumin preparations from the fasting sera of five normal individuals were assayed for insulin activity in vivo, in intact fed rats by the intraperitoneal assay of Rafaelsen et al.[18] Table 3 shows that the synalbumin preparations exhibited in vivo biologic effects similar to those of crystalline or "bound" insulin. They stimulated the incorporation of glucose carbon into muscle and adipose tissue glycogen and into the fat of the adipose tissue. Similarly, synalbumin preparations exerted insulin-like effects on isolated rat epididymal adipose tissue (figure 5).

## DISCUSSION

The present data indicate that "bound" insulin preparations inhibit both the biologic activity and the uptake of crystalline insulin by muscle. "Bound" insulin preparations also inhibited the uptake of crystalline insulin by isolated rat epididymal adipose tissue. Preliminary studies suggested that "bound" insulin did not inhibit the biologic activity of insulin on isolated rat adipose tissue. However, since "bound" insulin itself was active on adipose tissue, a quantitative evaluation of the data was difficult.

Inhibition of insulin uptake by isolated muscle, caused by "bound" insulin, could result from competition between crystalline insulin and "bound" insulin for binding sites on this tissue. Inhibition of crystalline insulin uptake by muscle, caused by preincubation with

TABLE 3

The effect of synalbumin preparations injected intraperitoneally into intact fed rats on the incorporation of radioactivity from glucose-u-C-14 into glycogen of muscle and glycogen and fat of adipose tissue

| Sample injected into each rat | Diaphragm glycogen CPM/gm. tissue | p< | Adipose tissue glycogen CPM/gm. tissue | p< | Adipose tissue fat CPM/gm. tissue | p< |
|---|---|---|---|---|---|---|
| Control (5 per cent HPA) | 2,700± 570 | — | 222±23 | — | 3,100± 800 | — |
| Synalbumin: | | | | | | |
| No. 1 (90 mg.) | 6,050± 830 | .01 | 1,098±315 | .02 | 6,200± 760 | .02 |
| No. 2 (100 mg.) | 4,000± 635 | N.S. | 903±241 | .02 | 5,270± 600 | N.S. |
| No. 3 (90 mg.) | 4,300± 417 | .05 | 769±145 | .01 | 5,500± 820 | N.S. |
| No. 4 (150 mg.) | 5,726± 892 | .02 | 1,361±319 | .01 | 6,100± 960 | 0.5 |
| No. 5 (110 mg.) | 5,900± 443 | .01 | 825±133 | .01 | 5,950± 860 | 0.5 |
| "Bound" insulin (1.0 mg.) | 12,150±1,116 | .001 | 700±120 | .01 | 10,600±1,200 | .001 |
| Crystalline insulin (1,000 μU.) | 13,426±1,680 | .001 | 2,320±310 | .001 | 10,200±1,360 | .01 |

Each synalbumin preparation, the "bound" insulin concentrate and the crystalline insulin was injected into six intact fed rats, along with 2 μc of glucose-u-C-14 (3.3 mc/millimole). Human plasma albumin was added into each synalbumin preparation and into the "bound" and crystalline insulin samples (50 mg./ml.). Values are means of the average counts per minute incorporated in the two epididymal pads or two hemidiaphragms per gram tissue ± S.E.M.
N.S. = not significant.

crystalline insulin (figure 2), also suggests a competition for insulin binding sites of the tissue. This possibility is consistent with the early data of Stadie et al.,[19-21] which demonstrated insulin binding on isolated muscle and the recent data of Rafaelsen et al.,[18] which provided evidence for a correlation between insulin binding to tissues and the biologic effects of this hormone on the tissues. Our finding that inhibition of insulin uptake by muscle, caused by "bound" insulin, was accompanied by inhibition of the biological activity of insulin on this tissue further supports the suggestion that insulin binding to tissue is important for the biologic effects of insulin on the tissue. The fate of insulin incubated with isolated muscle is unknown. It is possible that the insulin is eventually destroyed by proteolytic enzymes or modified by the tissue, and that the modified insulin is unreactive with the anti-insulin antisera and thus not detectable by immunoassay.

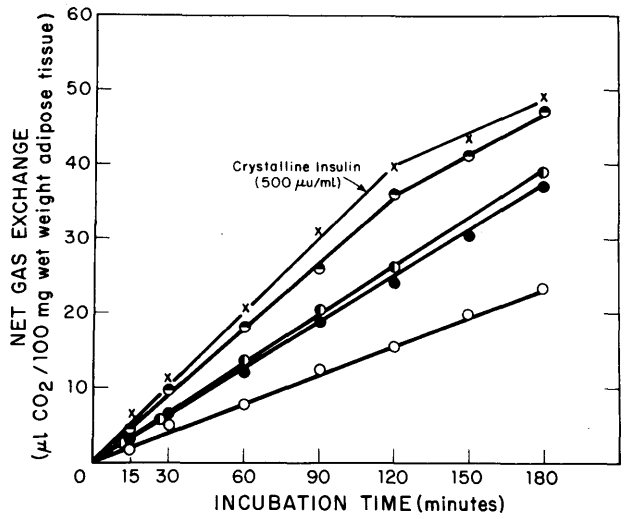


FIG. 5. Insulin-like activity of synalbumin preparations obtained from normal fasting human sera, on isolated rat epididymal adipose tissue.
Each synalbumin preparation contained about 30 mg. protein per ml. incubating medium corresponding to 1 ml. of original serum. The values are means of four incubations.

There are several similarities between "bound" insulin and synalbumin extracts from human sera. Synalbumin extracts, like "bound" insulin, were inactive on isolated rat muscle and they inhibited the uptake of crystalline insulin by isolated muscle and adipose tissue. In addition, as shown previously by Vallance-Owen and collaborators[10-13] and confirmed by us, they inhibited the biologic activity of insulin on isolated muscle. On the other hand, the synalbumin preparations exhibited insulin-like activity on isolated rat adipose tissue, a finding previously reported by Alp and Recant.[22] When synalbumin extracts were injected intraperitoneally into intact fed rats, they stimulated, as does "bound" insulin, the incorporation of glucose-u-C-14 into the glycogen of the muscle and adipose tissue and into the fat of the adipose tissue of the intact rats. It seems reasonable to suggest from these observations that synalbumin extracts from fasting sera may contain the bulk of "bound" insulin present in these sera. This is not surprising, since synalbumin is extracted from serum proteins by acid-ethanol, a proc-

ess which also enables the extraction of "bound" insulin from sera.

It is not clear as yet whether the presence of "bound" insulin in the synalbumin extracts can account fully for its insulin-inhibitory effect on isolated muscle. The synalbumin preparations we studied represented crude extracts containing about half the amount of protein in the original serum. It is possible that other substances besides "bound" insulin may contribute to the inhibitory effect of synalbumin.

The observation that "bound" insulin can inhibit the biologic activity of crystalline insulin on isolated muscle is in agreement with the earlier findings of Ensinck et al.[23-24] These investigators reported that "bound" insulin preparations could inhibit the activity of crystalline insulin on isolated muscle. Addition of adipose tissue extracts (ATE), which activate "bound" insulin on isolated muscle, could prevent the inhibitory effect of "bound" insulin. They suggested that "bound" insulin represents a mixture of "free" insulin with synalbumin—the synalbumin rendering the "free" insulin inactive on the isolated muscle. They also suggested the possibility that activation of "bound" insulin by ATE could result from the destruction of the synalbumin by ATE, thus unmasking the activity of "free" insulin. This interpretation is in disagreement with the fact that "bound" insulin is immunologically unreactive with anti-insulin antisera. If "bound" insulin were a mixture of "free" insulin with a synalbumin antagonist, then one could immunoassay the "free" insulin. It is also in disagreement with the fact that "bound" insulin exhibits a considerably higher molecular weight and a slower electrophoretic mobility than "free" insulin.[2] The possibility, therefore, emerges that the insulin antagonism exhibited by synalbumin extracts may be due, at least to some extent, to the presence of "bound" insulin in these extracts.

Insulin antagonism, caused by "bound" insulin, could be viewed as *secondary* and dependent on a number of factors such as the rate of insulin release by the pancreas, the rate of transformation of "free" to "bound" insulin in extrapancreatic tissues and the rates of utilization of "bound" insulin by the various tissues. Increased "bound" insulin levels accompanied by lack or slow rate of utilization of "bound" insulin by individual tissues could produce significant inhibition of insulin uptake by these tissues.

## ACKNOWLEDGMENT

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## ADDENDUM

Recent studies by the authors in collaboration with Dr. B. I. Posner suggest that "bound" insulin preparations can inhibit the uptake of crystalline insulin by the isolated perfused rat heart. When rat hearts from fed or fasted rats were perfused with crystalline insulin (200 μU./ml. in 3 per cent HSA), its concentration declined in the perfusion medium with time. In the presence of "bound" insulin concentrates (1 mg./ml. in 3 per cent HSA) the rate of disappearance of crystalline insulin from the perfusion medium was significantly reduced, "bound" insulin inhibiting the uptake of crystalline insulin by the perfused organ.

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## *Galactose Utilization in Young and Adult Rats*

The first step in the sequence by which galactose enters the pathway of glucose metabolism is catalyzed by the enzyme galactokinase. In this reaction, galactose-1-phosphate is formed from ATP and galactose. Consequently, any factors which alter the activity of the enzyme could affect the animal's ability to metabolize galactose.

P. Cuatrecasas and S. Segal (*J. Biol. Chem.* *240*:2382, 1965) investigated the effect of age on galactokinase activity in various tissues of both young and adult rats. In the adult rat the highest activity occurred in the liver, with significantly more activity in livers of the males. The intestine and kidney showed the next highest activities, the brain the third highest. There was relatively little activity in the sartorious muscle, but considerable in the diaphragm. The relatively high activities of the intestine, kidney, and brain are reasonable in view of their high metabolic activity.

Eleven days before birth, galactokinase activity in embryonic liver was approximately eight units. This increased to thirty-five units at birth, and then to forty units five days after birth. The activity declined gradually to approximately fifteen units at thirty days of age and then remained constant. The diet used in these studies was a commercial laboratory food.

Enzyme activity in intestine and kidney of the young animals was also greater than the adult levels, but differences were less pronounced than with liver enzymes. Kinetic studies and other tests indicated the probable occurrence of two liver enzymes which can phosphorylate galactose.

Feeding a diet containing 40 per cent galactose for five days had no effect on liver galactokinase activity in adult rats. However, feeding this diet for twenty days resulted in a significantly higher activity than in comparable animals fed a control diet. The experimental diet containing galactose was made by mixing 400 gm. galactose with 600 gm. of the laboratory food, which served as the control diet. The laboratory food was not a valid control diet, however, since it provided higher levels of protein, fat, and all other essential nutrients than did the diet containing galactose. However, the rise in galactokinase activity was assumed to result from the addition of galactose and was not prevented by the reduction in other essential nutrients.

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