Insulin I-131 Binding in Serum from Normal and Diabetic Subjects by Ultracentrifugation and Gel Filtration

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SUMMARY

Ultracentrifugation and gel filtration were used to test for specific insulin-I-131 binding protein in the serum of normal subjects and in patients with overt or potential diabetes.

Such technics permit study of sera at physiologic *p*H, with physiologic concentrations of exogenous insulin added. They also eliminate the interference of electrical fields and, in ultracentrifugation, the need for artificial supporting media.

Whereas a specific binding protein was readily demonstrable in the serum of insulin-treated patients with high antibody titers, there was no evidence of a protein capable of binding exogenous labeled insulin in normal subjects nor in the serum of subjects with various types of diabetes and potential diabetes.

Serum from subjects who have a hyper-response of insulin to glucose loads, possibly caused by peripheral antagonism to insulin action, did not bind significant amounts of insulin-I-131. DIABETES *14*:27-32, January 1965.

Serum insulin measured by immunochemical assay[1,2] is greater than normal in early maturity-onset diabetic patients after glucose loading[3,4] as well as in obese nondiabetic,[5] pregnant[6] and active acromegalic subjects.[1,2,5] These observations suggest that the presence of such large amounts of circulating insulin is required to overcome an inhibition to normal insulin function. One possible direct inhibitor is a specific insulin-binding protein capable of inactivating large amounts of circulating insulin.

When the distribution of insulin-like activity (ILA) in serum fractions separated by electrophoresis has been measured by biological assay, conflicting data have resulted. Randle and Taylor,[7,8] using ethanolized cellulose, detected ILA in the α_1 globulin and β-γ fractions of serum; Beigelman,[9] using preparative continuous flow paper electrophoresis, found ILA in the β globulin and in the intra-β-γ peaks; using starch column electrophoresis, Bollinger et al.[10] reported ILA in the α_1, β and γ globulins. These variable results may have been due in part to the nonspecificity of existing bioassays as well as the presence of antagonists influencing ILA activity.

The use of insulin-I-131 to study insulin-binding properties of serum has provided still more data showing insulin to have an electrophoretic mobility corresponding to many different protein fractions, depending on the technic used. Paper is not very useful as a medium for electrophoresis, since labeled insulin in low concentration is so firmly bound to the site of origin that a weak adsorption to proteins is obscured. While Bollinger et al.,[10] using a starch column, found the labeled insulin in the region of the β globulins, Randle and Taylor,[7] using ethanolized cellulose, found that labeled insulin traveled with a mobility corresponding to α_1 globulin and albumin. Mitchell[11] reported that the labeled insulin was located in the area of the α_2 and β globulins when electrophoresis was performed on cation-exchange resin paper.

Prout et al.[12] made use of the unique properties of agar gel, which allows substantial separation of the serum proteins, and at the same time applied the immunoelectrophoretic method to locate serum proteins associated with insulin-I-131. This technic showed that immunologically active insulin-I-131 was associated with an α_1 globulin. Clausen et al.,[13] however, using similar methods, found labeled insulin migrated with an α_2 and a β globulin.

To eliminate the interference of electrical current, artificial media, and unphysiologic *p*H, ultracentrifugation has been used to separate unbound from bound insulin-I-131 in serum of normal subjects.[14] The present study uses ultracentrifugation and gel filtration[15]

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to investigate this phenomenon in overt diabetic and potentially diabetic subjects, whose abnormally high levels of circulating insulin after glucose might be due to a special binding factor which was inactivating part of the circulating insulin.

MATERIALS AND METHODS

A. *Ultracentrifugation*

Porcine insulin-I-131 (Abbott), s.a. 250 to 350 microcuries per microgram, in a concentration of 40 μU. per milliliter, was incubated in serum for one hour at 4° C. or, in some cases, at 37° C. After incubation, six milliliter fractions of serum, undiluted or variously diluted with phosphate, bicarbonate or glycine buffers at *p*H 2 or 7.4, were spun from two to eighteen hours at 115,000 X g in a Beckman Spinco Model L ultracentrifuge. Then 1 ml. of the upper fraction was removed, counted and the concentration of radioactivity compared to the amount in an uncentrifuged aliquot. The result was expressed as "per cent radioactivity remaining." In order to provide enough serum to permit determinations in triplicate, the final conditions consisted of serum diluted 1:4 with bicarbonate buffer at *p*H 7.4, spun for three hours at 4° C. and 115,000 X g. The integrity of the labeled insulin before and after ultracentrifugation was established by hydrodynamic flow chromatography.[16]

The diluted supernatants were lyophilized and reconstituted to their original concentration and electrophoretic studies of the proteins were conducted at room temperature on Whatman 3 MM filter paper using 0.05 M barbital buffer at *p*H 8.6 for six hours. The proteins were stained with bromphenol blue, cut from the strip into their respective fractions, and eluted with acid alcohol. Optical densities were then compared to those of serum before centrifugation and results expressed as per cent protein remaining.

A group of eighteen subjects who had never been treated with insulin was studied while fasting and after glucose administration. It comprised four normal, five obese nondiabetic, two obese and two thin maturity-onset diabetic subjects, two with diabetes secondary to acromegaly or Cushing's syndrome, and three whose immediate family included two persons with diabetes or who had themselves delivered an infant weighing more than ten pounds.

Of the obese subjects, all were more than 30 per cent over ideal weight for their age, height and sex.[17] Included in this group were a number of subjects who had an exaggerated insulin response to glucose loading.[4,5] This was defined as levels of circulating insulin which, when measured by the immunochemical method of Grodsky and Forsham,[1] were more than 100 μU./ml. one and two hours after glucose administration. Normal values under these conditions in our laboratory did not exceed 100 μU./ml.[4]

To obtain blood containing antibodies, specimens were taken from six subjects with a history of insulin treatment, including three insulin resistant diabetics and three juvenile diabetics. Antibody titers were determined by the method previously described.[18]

B. *Gel filtration*

Sephadex G-100* was washed three times in 0.9 per cent NaCl solution (*p*H 7.4), and finer particles of the suspension were removed by decantation. After the gel had swelled for one hour a column 27 cm. long and 0.9 cm. in diameter was prepared. Flow rate was 0.5 ml. per minute.

Serum obtained from the four normal subjects, one insulin resistant subject, and three with excessive serum insulin response, or samples of 5 per cent albumin and normal saline were incubated for one hour at 37° C. with 40 μU./ml. of porcine insulin-I-131. Then 0.1 ml. of the sample was applied to the column and during elution with saline the effluent was recovered in 1 ml. portions. Proteins were measured spectrophotometrically at 280 mμ and radioactivity was determined in a well scintillation counter.

RESULTS

A. *Ultracentrifugation*

Since the denatured products in commercial insulin varied from 5 to 15 per cent between preparations, flow chromatography was performed before centrifugation, and the ratio of intact insulin to denatured products at zero time was compared to the ratio after three hours of centrifugation.

In fourteen experiments we noted that if the ratio of intact insulin-I-131 to the degradation products in the supernatant was arbitrarily assigned a value of 1.00, the ratios remained essentially unchanged after centrifugation, ranging from 0.92 to 1.06. This indicated that there was no denaturation and no significant preferential sedimentation of either the intact insulin or the denatured products under these conditions.

At a physiological *p*H of 7.4 the sedimentation of insulin-I-131 was found to be a direct function of the time of centrifugation (figure 1), with 60 to 75 per

*Pharmacia, Uppsala, Sweden.

cent of the original concentration of insulin-I-131 remaining in the upper fraction of normal sera, 0.9 per cent saline, or 5 per cent albumin solutions after three hours of centrifugation at 115,000 X g.

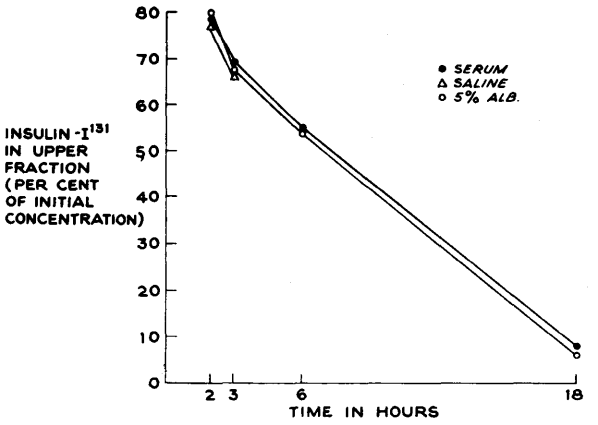


FIG. 1. The effect of time of ultracentrifugation at 115,000 X g on sedimentation of insulin-I-131 at 4° C. in serum, 0.9 per cent saline and 5 per cent albumin solutions.

Dilution of serum to 25 per cent, 5 per cent or 0.5 per cent with phosphate, bicarbonate, or glycine buffers or with saline solution alone did not alter the distribution of insulin (figure 2). While the sedimentation of insulin did not vary from *p*H 7.4 to *p*H 9.8, increased sedimentation at *p*H 2.0 was noted in undiluted serum and in 5 per cent albumin and 0.9 per cent saline solutions (figure 3).

Sera selected for their lack of hemolysis, and in which insulin degradation was minimal even at elevated temperatures, were incubated and centrifuged at 37° C. In all cases results were comparable to those in studies done at 4° C.

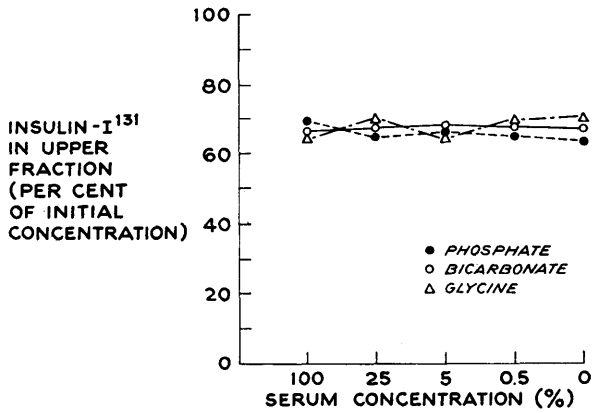


FIG. 2. The effect of various dilutions of serum, using 0.2 M glycine, 0.1 N sodium bicarbonate and 0.05 M phosphate buffers (pH 7.4) on the sedimentation of insulin-I-131 at 4° C. and 115,000 X g for three hours.

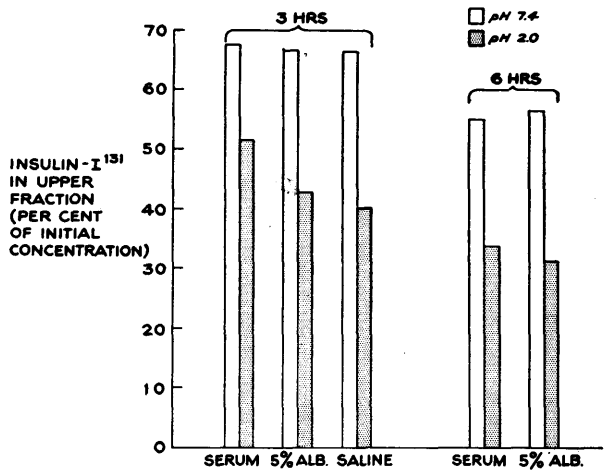
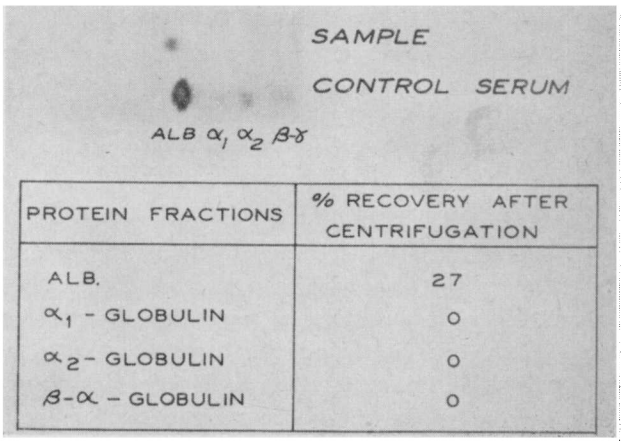


FIG. 3. The effect of pH on sedimentation of insulin-I-131 added to undiluted serum, 5 per cent albumin and 0.9 per cent saline, at 4° C. and 115,000 X g for three and six hours.

Figure 4 shows the effectiveness of the final conditions used for the studies of normal and pathologic serum in separating free insulin from insulin bound to serum globulins. Despite the fact that 70 per cent of the original concentration of added labeled insulin remained in the upper fraction after centrifugation of a 1:4 dilution of serum with bicarbonate buffer, at *p*H 7.4, 4° C. and 115,000 X g, only 27 per cent of the albumin and no detectable globulin remained. Thus, free insulin remained in the supernatant under conditions in which all insulin bound to globulins and most



PROTEIN FRACTIONS	% RECOVERY AFTER CENTRIFUGATION
ALB.	27
α_1 - GLOBULIN	0
α_2 - GLOBULIN	0
β-α - GLOBULIN	0

FIG. 4. Electrophoretic separation of serum proteins in the upper 1 ml. fraction after ultracentrifugation. Experimental conditions of ultracentrifugation consisted of the use of serum diluted 1:4 with 0.1 N sodium bicarbonate, spun at 4° C. using a force of 115,000 X g for three hours. The supernatant was lyophilized and reconstituted to its original concentration and subjected to electrophoresis at pH 8.6 for six hours.

of that associated with albumin would be removed.

The technic demonstrated insulin binding to globulins in sera containing insulin antibodies. Figure 5 shows that 65 to 75 per cent of the added labeled insulin remained in the upper fraction of samples of 5 per cent albumin, normal serum, or saline alone after ultracentrifugation, but most of the insulin was removed with the globulin fraction from the serum of patients with antibody titers. In one patient recently given insulin who had no antibody, there was no evidence of insulin binding. In others with antibody titers a general correlation was seen between the sedimentation of the insulin-I-131 and the antibody titer. The 10 to 15 per cent remaining radioactivity in the supernatant of serum of patients with high antibody titers probably represented degraded, immunologically inactive insulin products retaining the chromatographic properties of insulin.

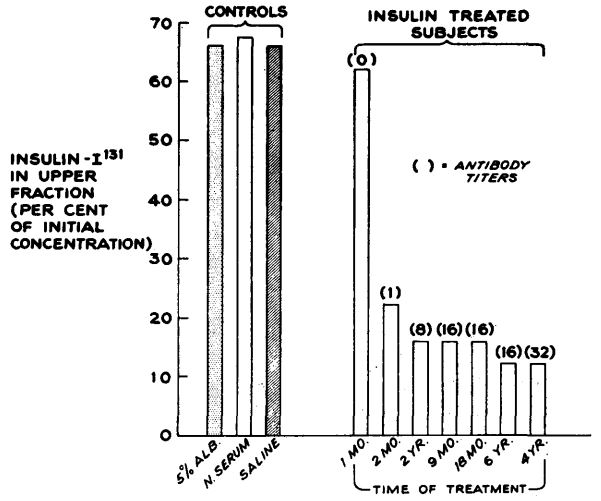


FIG. 5. The effect of antibody on the sedimentation of insulin-I-131 under the experimental conditions described in figure 4.

The fourteen diabetic or potentially diabetic subjects studied included eight with elevated serum insulin levels measured by immunochemical assay after glucose loading. The results in figure 6 indicate that when all plasma globulins were centrifuged into the bottom layers, 65 to 75 per cent of the concentration of labeled insulin remained in the upper fraction—values identical to those observed in the normal controls and with albumin or saline alone. There was no evidence that the serum of normal subjects or those with excessive serum insulin response bound any appreciable amount of insulin-I-131.

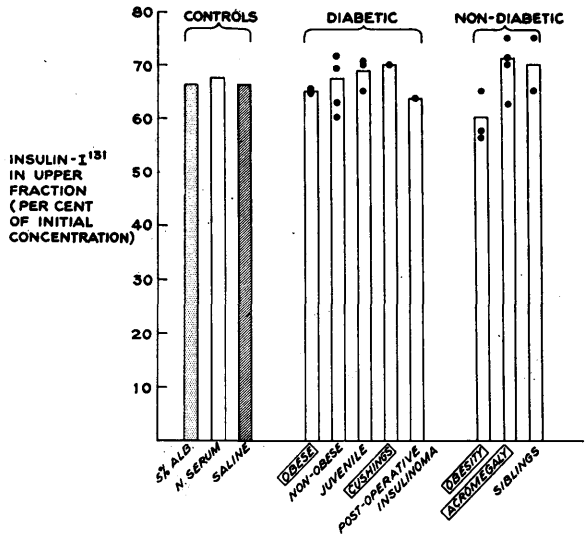


FIG. 6. Insulin-I-131 remaining in upper fraction in serum of subjects not treated with insulin. Conditions of ultracentrifugation were outlined in figure 4. Dots represent patients. |═══| ═ subjects with proved hyper-response of serum insulin to glucose.

B. *Gel filtration*

A representative example of the distribution of protein and insulin-I-131 in serum from a normal subject after gel filtration is shown in figure 7. Peak I contained the bulk of serum proteins, whereas only a small per cent of "protein" appeared in the after portion, Peak II. The latter may well have included tyrosine and tyrosine peptides, which are also measured by absorbency at 280 mμ. The major portion of the radioactivity was found in Peak II and, in this technic, represents unbound insulin. The small proportion of radioactivity accompanying the proteins in Peak I could account for no more than 4 to 6 μU./ml., even if all of it were intact insulin.

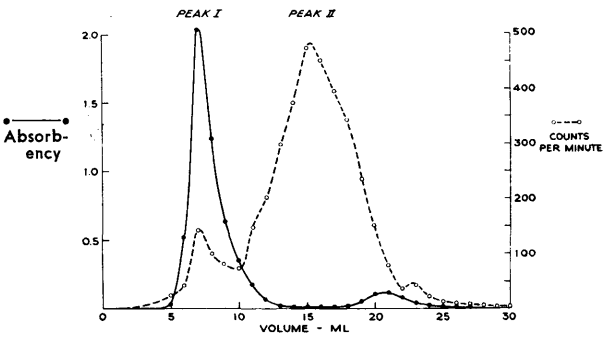


FIG. 7. Distribution of protein and radioactivity of normal serum incubated with insulin-I-131 after gel filtration using Sephadex G-100.
●———● Absorbency at 280 mμ
O - - - - - O Radioactivity in counts per minute

Table 1 presents the data of ten experiments. In all sera studied, Peak I contained 95 to 99 per cent of the serum proteins. In a patient with insulin resistance whose antibody titer was 1:32, 91 per cent of the radioactivity was in Peak I, whereas in four normal subjects only 9 to 14 per cent of the radioactivity was in this peak containing the serum proteins. When insulin hyper-responders were studied by this method, essentially the same distribution of radioactivity as in normal subjects was found.

The small per cent of isotope in Peak I did not represent specific binding of insulin to serum proteins since a similar distribution occurred after gel filtration of radioactive insulin incubated with 5 per cent albumin or even saline alone.

TABLE 1

The association of insulin-I-131 with serum proteins as measured after gel filtration

Serum incubated with insulin-I-131	Per cent radioactivity Peak I*	Peak II
Insulin resistant patient	91	9
Normal subject	9	91
Normal subject	12	88
Normal subject	11	89
Normal subject	14	86
Insulin hyper-responders	14	86
Insulin hyper-responders	12	88
Insulin hyper-responders	13	87
Albumin 5 per cent	15	85
Saline 0.9 per cent	12	88

*Peak I = Initial fraction from column containing 95 to 99 per cent of protein content of serum.

DISCUSSION

Ultracentrifugation and gel filtration provide methods for measuring the association of labeled insulin with serum globulins and eliminate the electrical field and unphysiological *p*H used in electrophoretic methods. Ultracentrifugation also eliminates the need for artificial supporting media. An unphysiological temperature of 4° C. was used to minimize the degradation of insulin that occurs in serum, particularly when some hemolysis is demonstrable.[19] However, ultracentrifugation of virtually unhemolyzed serum at 37° C. showed the same results, indicating that temperature was not an important factor.

Sedimentation of insulin varied in response to extremes of *p*H change. Since the increased sedimentation at *p*H 2 was similar in saline, serum, and albumin solutions, it is probably a property of the insulin molecule itself, rather than the effect of its binding to a serum protein.

Validation of these technics for measuring binding is provided by the positive results seen in insulin-treated patients who had measurable antibody titers. Only for this group is there universal agreement that insulin-binding globulins are present.[12,16,20]

Our results confirm Berson and Yalow's finding[14] that in normal subjects there is no evidence of a serum protein carrier of added insulin-I-131 after ultracentrifugation. In addition, untreated subjects with overt and potential diabetes mellitus, including many with excessive circulating levels of insulin after glucose, did not differ from the normal group.

There was no evidence of an insulin-binding protein by gel filtration. These findings suggest that in subjects with excessive serum insulin response binding is not a factor in the production of abnormally high serum insulin levels.

Randle and Taylor have reported the association of added insulin-I-131 with albumins as well as with α_1 globulins.[7] While the globulins were entirely removed during ultracentrifugation, only 73 per cent of the albumin was. It is unlikely, however, that the albumin played any important role in insulin binding, since sedimentation patterns for insulin-I-131 were identical in buffered salt solutions free of albumin, as in whole serum and in serum after sedimentation of most of the albumin.

Prout et al.[12] and Clausen et al.,[13] using immunoelectrophoresis in agar gel, have supplied direct evidence that insulin is bound to serum globulins. However, this association of insulin-I-131 with globulins may be an artifact of the technic which requires unphysiologic conditions such as elevated *p*H and a charged field.

Recent interest in the possibility that insulin is brought into a complex with a basic protein, perhaps by the liver, has been stimulated by the work of Antoniades[21] and Samaan et al.[22] Although this study presents evidence against the existence of a protein that binds insulin-I-131 in the serum of patients not previously treated with insulin, it does not exclude the possibility that some endogenous insulin exists in vivo as part of a protein complex that cannot be duplicated with exogenous insulin-I-131. It is of interest that the suggested high levels of bound insulin in serum can not yet be demonstrated by direct immunochemical assay[23,24] or immunochemical assay of various types of acid alcohol extracts of serum.[1,5]

ACKNOWLEDGMENT

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