Determination of Free and Total Insulin and C-Peptide in Insulin-treated Diabetics

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SUMMARY

Serum levels of free and total insulin as well as total C-peptide immunoreactivity (C-peptide and proinsulin) and C-peptide were measured in insulin-treated diabetics with circulating insulin antibodies by the addition of polyethylene glycol (PEG) before and after acidification. PEG resulted in complete precipitation of insulin antibodies from serum and made it possible to measure free insulin in the supernatant. Incubation of serum at 37° C. for two hours before addition of PEG resulted in values for free insulin that probably resembled the in-vivo levels most closely. The same method could also be used to remove proinsulin bound to circulating insulin antibodies and permitted the measurement of C-peptide in the supernatant.

Clinical studies using this approach indicate that combined measurements of serum free and total insulin and C-peptide provide information that is helpful in understanding the contribution of endogenous and exogenous insulin to the course and metabolic control of insulin-requiring diabetic patients. DIABETES *26*:22-29, January, 1977.

Diabetic patients who have been treated with commercial preparations of bovine-porcine insulin for periods longer than a few weeks develop circulating insulin antibodies. In these patients, endogenously secreted and exogenously administered insulin circulate in both the free and antibody-bound state, and the antibodies complicate the accurate measurement of serum insulin levels by radioimmunoassay.[1]

Since the development of a radioimmunoassay for human C-peptide,[2,3] which is secreted from pancreatic beta cells in equimolar concentrations with insulin,[4] this technique has provided a simple method for monitoring beta-cell function in insulin-requiring diabetics despite the presence of circulating insulin antibodies.[5,6] Although insulin antibodies do not interfere with the human C-peptide assay, they do bind endogenously secreted proinsulin by way of its insulin moiety and greatly retard its clearance from the circulation.[3] Because most C-peptide antisera react to a greater or lesser extent with human proinsulin, this peptide may become a major determinant of serum total C-peptide immunoreactivity.[7,8] Therefore, for the accurate assessment of beta-cell secretory function, antibody-bound proinsulin should be separated from C-peptide prior to assay.

In an attempt to solve these problems, we have evaluated the utility of the polyethylene glycol precipitation method, which was introduced by Nakagawa et al.[9] to remove insulin antibodies from sera. The method was then used to follow serum free and total insulin and C-peptide levels in insulin-requiring diabetic patients in a variety of clinical circumstances.

MATERIALS AND METHODS

Precipitation of Insulin Antibodies in Serum with Polyethylene Glycol (PEG)

Free insulin and C-peptide: 0.3 ml. 25 per cent (w/w) aqueous PEG (Carbowax 6000) solution (chilled to 4° C.) was added to 0.3 ml. serum, mixed vigorously, and centrifuged at 4° C. The supernatant was assayed for insulin and/or C-peptide.

Total insulin: 0.06 ml. 1.0 N HCl was added to 0.3 ml. serum. After one hour of incubation at room temperature, 0.42 ml. 25 per cent PEG solution (ch lled at 4° C.) was added and the mixture was shaken vigorously; 0.06 ml. 1.0 N NaOH was then added. After thorough mixing, the supernatant, containing all the insulin, was separated by centrifugation at 4° C. and assayed.

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Accepted for publication July 14, 1976.

Immunoassay

A modification of the double-antibody method of Morgan and Lazarow[10] was used for the insulin and C-peptide radioimmunoassays. The standard dilutent was 0.133 M borate buffer (pH 8.0) containing 0.5 per cent bovine serum albumin.

Insulin radioimmunoassay: First reaction (three days at 4° C.); to 1.0 ml. buffer containing human insulin standard, 0.1 ml. serum or 0.1 ml. PEG supernatant, 0.1 ml. ^{125}I-porcine insulin, and 0.1 ml. guinea pig antiporcine insulin serum (final dilution 1:300,000) were added. When the PEG supernatant was assayed, 0.1 ml. 12.5 per cent PEG solution was added to the standard tubes to simulate the PEG concentration in the sample tubes. Second reaction (three days at 4° C.); 0.1 ml. normal guinea pig serum (1:300) and 0.1 ml. (1:5) rabbit antiserum made to guinea pig serum were added. After centrifugation at 4° C., the precipitates were counted.

Standard curves of porcine and bovine insulin were comparable (within 5 per cent) to that of human insulin in this assay system.

C-peptide radioimmunoassay: First reaction (three days at 4° C.); to 0.8 ml. buffer containing human C-peptide standard, 0.1 ml. serum or 0.1 ml. PEG supernatant, 0.1 ml. ^{125}I-tyrosylated C-peptide, and 0.1 ml. rabbit antihuman C-peptide serum (final dilution 1:15,000) were added. When the PEG supernatant was assayed, 0.1 ml. 12.5 per cent PEG solution was added to the standard tubes. Second reaction (three days at 4° C.); 0.1 ml. normal rabbit serum (1:100) and 0.1 ml. sheep antirabbit globulin serum (1:20) were added to the assay tubes. After centrifugation at 4° C., the precipitates were counted. Human proinsulin reacts approximately 1.1 to 1.8 times less well than C-peptide on a molar basis at peptide concentrations below 0.3 p mole/tube with this particular antiserum; i.e. in this range, 0.11 to 0.18 pmole of proinsulin is needed to displace labeled C-peptide to the same degree as seen with 0.10 pmole C-peptide.

Antibodies in the PEG Supernatant

To confirm whether complete precipitation of the antibodies was affected with PEG, the supernatants, which were used for free insulin determinations, were checked as follows: 0.1 ml. ^{125}I-porcine insulin was added to 1.0 ml. buffer containing 0.1 ml. of the PEG supernatant or 0.05 ml. of the extracted serum. The tubes were adjusted to include the same concentration of PEG. After three days' incubation at 4° C., free ^{125}I-porcine insulin was adsorbed with dextran-coated charcoal.[11]

Gel Filtration of Sera

Sera from insulin-treated diabetics were fractionated on 1 × 50 cm. columns of BioGel P-30 (Bio-Rad Laboratories, Richmond, Calif.), 100-200 mesh equilibrated in 3 M acetic acid and calibrated with proinsulin, insulin, and ^{125}I-tyrosylated C-peptide markers. The fractions were evaporated to dryness under reduced pressure and, after buffer was added, assayed for insulin or C-peptide immunoreactivity.[2]

Statistical Analysis

Results are expressed as mean ± S.E.M. unless stated otherwise.

RESULTS

Free and Total Insulin

Comparison of insulin concentration in serum and PEG supernatant in normal subjects. In order to determine whether PEG interfered with the insulin assay and the recovery of insulin after precipitation of serum proteins, PEG was added to aliquots of sera from normal subjects (n = 16) before and after acidification. Insulin concentrations in the PEG supernatants obtained from these two procedures were compared with the insulin values obtained by the direct assay of unextracted serum. The standard curve with added PEG differed slightly from that without PEG. In the presence of PEG, a higher percentage of tracer was bound to antibodies at each concentration of the insulin standard. However, when the standard curve with PEG was used for the assay of PEG supernatants, the three values for serum insulin were comparable (figures 1 and 2). The insulin values in sera processed according to the method for measuring free insulin ranged from 81 to 119 per cent (106 ± 3 per cent) of the direct assay value, while sera treated according to the procedure for measuring total insulin averaged 110 ± 3 per cent.

Recovery of insulin following PEG precipitation. ^{125}I-porcine insulin or unlabeled human insulin (14.7-57.3 μU.) was added to normal serum. After addition of PEG the supernatant was counted or assayed. The recovery of ^{125}I-insulin in the supernatant (n = 5) was 91 ± 1 per cent, while that of cold insulin (n = 6) was 95 ± 2 per cent.

In parallel experiments, human insulin (58-231 μU.) was added to serum from insulin-treated diabetic patients and the mixture precipitated with PEG after acidification. The per cent recovery in the PEG supernatant (n = 5) was 83 ± 11 per cent.

Effect of time between addition of PEG and centrifugation on the free and bound hormone. ^{125}I-porcine insulin or ^{131}I-porcine proinsulin was added to aliquots of

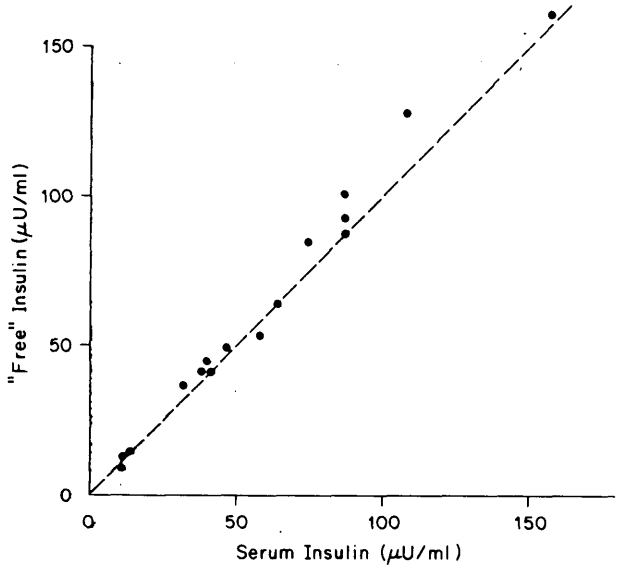

FIG. 1. Comparison of insulin concentration in serum and PEG supernatant in normal subjects. Sera were treated according to the procedure for measuring free insulin. The insulin value in the PEG supernatant ("free" insulin) was compared with the direct assay value. The correlation coefficient is r = 0.99.

serum containing insulin antibodies. After two days' incubation at 4° C., PEG was added and the mixtures were allowed to stand at 4° C. for zero to 90 minutes. The tubes were then centrifuged and the supernatants,

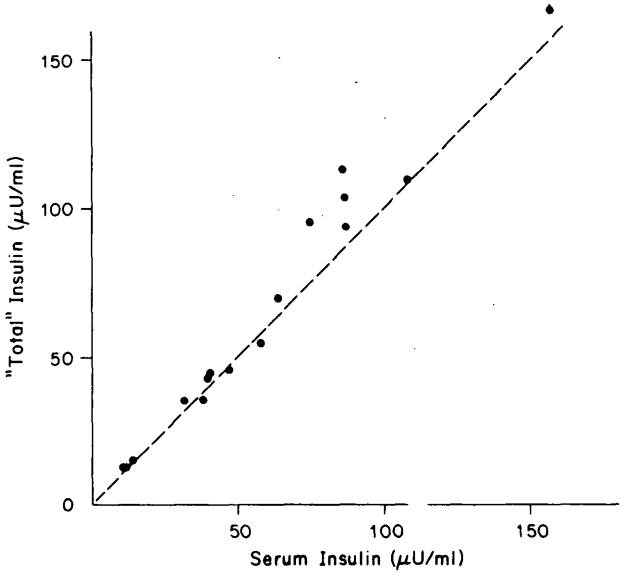

FIG. 2. Comparison of insulin concentration in serum and PEG supernatant in normal subjects. Sera were processed according to the procedure for measuring total insulin. The insulin value in the PEG supernatant ("total" insulin) was compared with the direct assay value. The correlation coefficient is r = 0.99.

containing free ^{125}I-insulin or ^{131}I-proinsulin, were counted. The results indicate that a delay of centrifugation for as long as 90 minutes does not affect distribution of counts between the supernatant and precipitate (table 1).

TABLE 1

Effect of time between addition of PEG and centrifugation on the percentage free and bound hormone

Time (mins.)	% Free ^{125}I-insulin	% Free ^{131}I-proinsulin
0	59.7%	54.8%
15	60.9	53.9
30	60.1	53.8
60	61.2	54.5
90	60.1	54.9

Antibodies in the supernatant. Sera or PEG supernatants from 20 insulin-treated patients and six normal subjects were incubated with ^{125}I-insulin for 72 hours. The per cent free ^{125}I-insulin was then assessed in each sample by the addition of dextran-coated charcoal, which adsorbed the non-antibody-bound hormone (figure 3). In normal sera or their PEG supernatants, almost 100 per cent of the added radioactivity was adsorbed by dextran-coated charcoal. On the other hand, in unextracted sera from insulin-treated diabetics, the values ranged from 27 to 100 per cent. After PEG precipitation, 98 ± 0.5 per cent of the radioactivity was adsorbed by charcoal in 19 of these 20 samples. A low level of residual antibodies remained in one sample (figure 3).

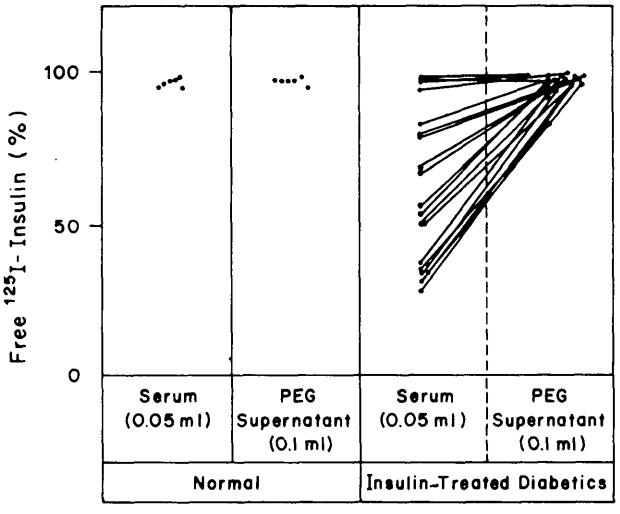

FIG. 3. Presence of residual insulin antibodies in the PEG supernatant. Serum was incubated with ^{125}I-insulin before and after PEG precipitation, and the per cent free ^{125}I-insulin was assessed in each sample by the addition of dextran-coated charcoal.

Comparison of total insulin measured by gel filtration and PEG precipitation. Insulin and proinsulin were dissociated from antibodies by adding 3.M acetic acid to serum, and the mixture was gel-filtered on BioGel P-30 columns. After immunoassay of the fractions, the sum of the proinsulin and insulin peaks was compared with the total insulin value obtained by the PEG method. As shown in figure 4, both methods gave comparable results.

Effect of temperature on the binding of insulin to its antibodies. Serum samples from insulin-treated diabetics, which had been stored at −20° C. for as long as six months, were studied. As soon as the serum thawed, an aliquot was precipitated with PEG for the determination of free insulin. Additional aliquots of serum were incubated at 37° C. for two, five, nine, and 16 hours and precipitated with PEG, and the insulin concentrations in the supernatants were measured. The free insulin concentrations in the majority of sera initially increased and reached a plateau after two-to-five-hour incubation (figure 5). In a few samples, the free insulin concentration increased or decreased with longer incubation times. This was particularly striking in one patient with antibody-mediated insulin resistance, whose free insulin levels continued to increase even after 16 hours' incubation. The mean free insulin values (excluding this particular patient) before incubation and after two, five, nine, and 16 hours' incubation at 37° C. were 12.7 ± 2.3, 28.4 ± 4.5, 34.2 ± 4.4, 37.4 ± 4.6, and 33.5 ± 4.6 μU./ml. (n=13), respectively. There was no significant difference between the five-, nine-, and 16-hour values, but the two-hour value was significantly lower than those obtained with longer periods of incubation ($p < 0.02$).

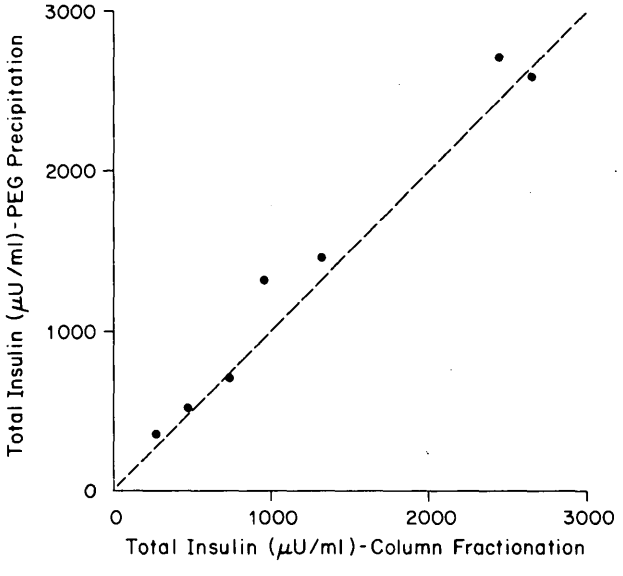

FIG. 4. Comparison of total insulin measured by gel filtration and PEG precipitation. The correlation coefficient is r = 0.99.

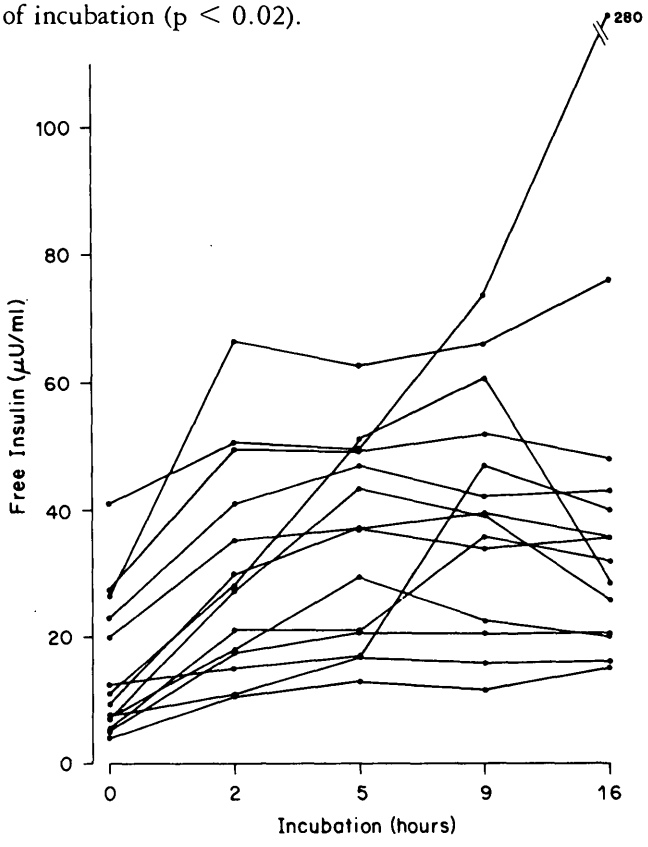

FIG. 5. Effect of temperature on the binding of insulin to its antibodies. After storage at −20° C., thawed aliquots were incubated at 37° C. for 0, 2, 5, 9, and 16 hours and precipitated with PEG, and the free insulin concentrations in the supernatants were measured.

In additional experiments, blood was drawn from insulin-treated diabetics (n=5) and plasma (collected in heparinized tubes) or serum was separated by centrifugation at 37° C. An aliquot of serum or plasma, maintained at 37° C., was immediately precipitated with PEG. Following storage of the serum at −20° C. for two to four weeks, aliquots were thawed and, after incubation at 37° C. for zero to five hours, precipitated with PEG. The supernatants were assayed for insulin (table 2). The free insulin concentrations in the stored samples incubated for two hours at 37° C. were similar to those measured in freshly drawn serum or plasma maintained at 37° C.

Reproducibility of PEG precipitation method. Aliquots of serum from insulin-treated patients (n=8) were incubated at 37° C. for two hours and precipitated with PEG for free insulin determinations on three different

TABLE 2

	Free insulin (μU./ml.)				
	Freshly drawn plasma or serum maintained at 37° C.		Stored serum (−30° C., 2-4 weeks). Before precipitation aliquots were incubated at 37° C. for 0 to 5 hours		
Patient	Plasma	Serum	0 hour	2 hours	5 hours
A	13.4	10.7	7.7	10.3	16.7
B	4.2	3.9	2.4	5.5	8.3
C	31.0	28.8	5.9	26.4	19.9
D	19.0	20.1	19.9	19.0	17.5
E	—	28.0	14.5	28.8	—

days. The insulin concentration was measured in three different assays. The coefficient of variation of the three determinations was 10.0 per cent.

C-peptide

Comparison of C-peptide concentrations in normal sera before and after PEG precipitation. C-peptide values in sera (n=37) obtained from control subjects were determined by direct assay. Aliquots of these sera were precipitated with PEG and the supernatants assayed for C-peptide. Both assays gave comparable results (figure 6). The supernatant value was 99 ± 3 per cent of that obtained by direct assay. In contrast to the findings with the insulin assay, the standard curve of C-peptide in the presence of PEG was superimposable on the curve carried out in buffer alone.

Recovery of C-peptide after PEG precipitation. The recovery of C-peptide in the PEG supernatant was examined after adding ^{125}I-tyrosylated human C-peptide and unlabeled human C-peptide to serum. In normal serum almost 100 per cent of added labeled C-peptide was recovered in the supernatant (n=5). The recovery of 1.2 to 2.3 ng. of unlabeled C-peptide in normal (n=6) and insulin-treated diabetics' (n=6) sera was 88 ± 5 per cent.

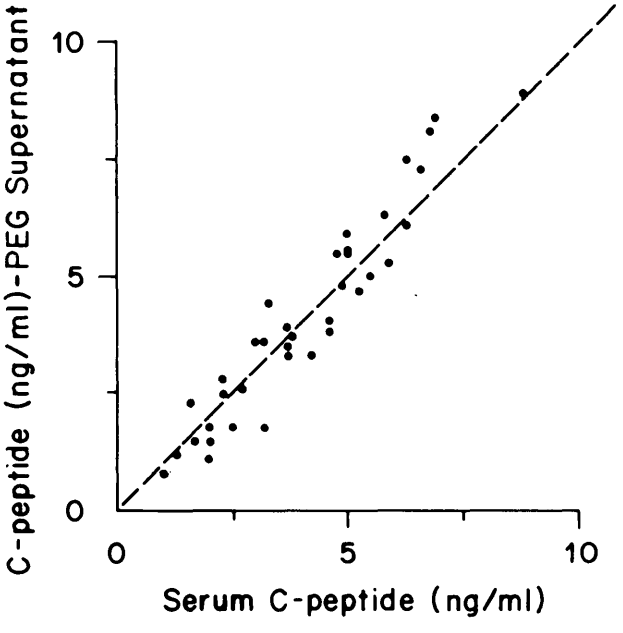

FIG. 6. Comparison of C-peptide concentration in unextracted sera and PEG supernatants in normal subjects. C-peptide values in the PEG supernatant were compared with the direct-assay value. The correlation coefficient is r = 0.95.

Comparison of C-peptide concentrations determined by gel filtration and PEG precipitation. Seven sera from insulin-treated diabetic patients were assayed directly. The results showed high levels of C-peptide immunoreactivity (CPR) in all the samples (9.2 ± 1.3 ng./ml. n=6), the value in one serum exceeding 20 ng./ml. Gel filtration of sera on BioGel P-30 columns equilibrated in 3 M acetic acid showed two peaks of C-peptide immunoreactivity corresponding to markers of proinsulin and C-peptide. Antibody-bound proinsulin was dissociated by the addition of 3 M acetic acid and eluted in the proinsulin region. The contribution of proinsulin to serum total C-peptide immunoreactivity (sum of proinsulin and C-peptide peaks) ranged from 11 to 96 per cent. The C-peptide concentration determined by gel filtration was compared with that measured by PEG precipitation. The two methods gave comparable results (figure 7).

In 17 additional sera from fasting patients with circulating insulin antibodies, total CPR concentrations were determined by direct assay of unextracted sera. The values ranged between 2.1 and 28.7 ng./ml. (7.5 ± 1.6 ng./ml.). Antibody-bound proinsulin was then precipitated with PEG,and the C-peptide concentration was measured in the supernatant (figure 8). The free C-peptide values ranged from 0.5 to 4.3 ng./ml. (1.5 ± 0.3 ng./ml.).

Clinical Application

Using these techniques, we have initiated a number of clinical studies, which will be published in detail elsewhere. However, the following examples are given in order to indicate the type of investigation which can now be undertaken.

Diurnal changes of plasma glucose and serum levels

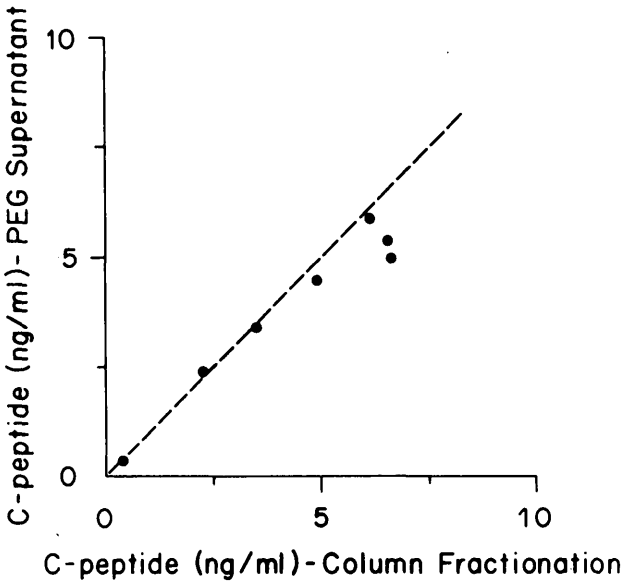

FIG. 7. Comparison of C-peptide concentrations determined by gel filtration and PEG precipitation in sera of insulin-treated diabetics. The correlation coefficient is r = 0.98.

of free and total insulin and CPR were measured in nine juvenile-onset diabetic patients, who received a single dose of insulin (NPH or mixed regular-NPH) before breakfast.[12] Serum CPR was very low and did not rise significantly during the 24 hours. Mean concentrations of fasting free and total insulin were 10 μU./ml. (range: 2.5 to 25.2 μU./ml.) and 1,765 μU./ml. (range: 193 to 3,911 μU./ml.), respectively. The time of the day at which serum free and total insulin levels reached peak values varied considerably from patient to patient. However, the peak serum free insulin levels correlated well with the maximal fall in plasma glucose. Analysis of these findings improved our understanding of the reasons for poor control in some of the patients and suggested changes in the choice of insulin therapy.

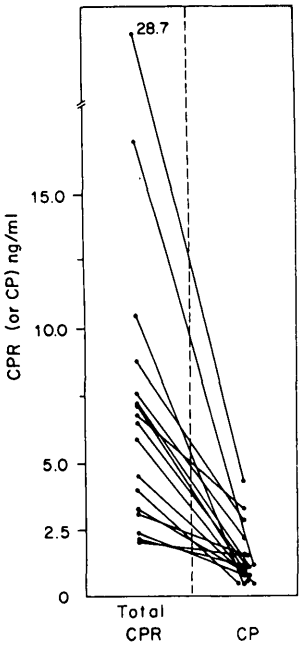

FIGURE 8

Comparison of total CPR and C-peptide concentrations in sera containing insulin antibodies. The values determined by direct assay (total CPR) were compared with those measured after PEG precipitation (C-peptide). The sera were obtained from insulin-treated diabetics, a patient with insulin autoimmune syndrome, a patient with factitious hypoglycemia, and newborn babies from insulin-treated diabetic mothers.

Serum free and total insulin, CPR, and C-peptide values were measured hourly over 24 hours in seven pregnant diabetic women (White's classes B through D), who were maintained in good metabolic control by insulin therapy.[13] The high CPR concentrations observed in the mild diabetics was shown to be due to high levels of antibody-bound proinsulin. However, their C-peptide concentrations measured after precipitation of the proinsulin-antibody complex by PEG were comparable to those of control women. The mean free insulin levels over 24 hours in these well-controlled diabetics were somewhat higher than in the controls (42.4 ± 5.1 μU./ml. vs. 24.4 ± 2.0 μU./ml.), and the reasons for this difference are now being studied.

DISCUSSION

Since Desbuquois et al.[14] observed that an aqueous solution of PEG precipitated antibody-bound peptide hormones, whereas the unbound (or free) hormone remained in solution, this method has been successfully used for separating free from antibody-bound hormone in various radioimmunoassays. The method has proved useful because, under controlled conditions, complete precipitation of antibody-bound hormone is effected with little or no coprecipitation of free hormone. Nakagawa et al.[9] subsequently applied this technique for the measurement of free and total insulin levels in sera from insulin-treated diabetic patients. In the present study we have confirmed the usefulness of this approach and, in addition, defined some of the variables that must be taken into account in the interpretation of the free and total insulin values.

Following precipitation of insulin antibodies with PEG, small volumes (0.1 ml.) of the supernatant could be assayed directly with only minor interference in the double-antibody insulin immunoassay. Addition of a similar volume (0.1 ml.) of PEG to the standard curve resulted in essentially full recovery of endogenous insulin in sera without insulin antibodies, as well as added labeled and unlabeled insulin in sera taken from both control and diabetic subjects. Satisfactory separation of insulin bound to antibodies following acidification with hydrochloric acid was indicated by the similar total insulin values obtained by

this method and gel filtration of sera in 3 M acetic acid. Furthermore, significant quantities of insulin antibodies were not detected in the supernatant after addition of PEG in 19 of 20 samples. However, as the PEG supernatant of one serum which had a high concentration of insulin-binding antibodies showed binding of ^{125}I-insulin, it might be advisable to check for the presence of residual antibodies in each sample.

It is unlikely that the free insulin concentration determined in serum samples that have been stored at −20° C. and thawed at room temperature accurately reflects the true serum free insulin levels in vivo. Therefore, the effect of temperature of the binding of insulin to its antibodies was examined. Incubation of serum at 37° C. prior to adding PEG resulted in an increase in free insulin levels, which reached a plateau after two to five hours. Further studies suggested that the two-hour incubation values reflected the circulating free insulin levels most closely. Although the relationship between the incubation time and the free insulin value varied slightly from serum to serum, we decided on the basis of these results, as well as for practical considerations, to incubate all serum samples for two hours at 37° C. before PEG precipitation.

Other investigators have described a number of techniques for measuring circulating insulin concentrations in insulin-treated diabetic patients.[1,15-19] Their results for total insulin are similar to those we determined by the PEG precipitation method in juvenile-onset diabetics.[12] For example, Heding described an acid-ethanol extraction method[16] for the determination of total insulin in insulin-treated diabetics. The fasting total serum insulin was 392 μU./ml. (range: 6 to 4,374 μU./ml.) in 169 insulin-treated patients. The fasting free insulin levels in 57 insulin-treated diabetics ranged from 0 to 20 μU./ml. in the study by Nakagawa et al.[9] Again, our findings in similar patients fall into the same range.[12] It should be noted that we were able to adapt the double-antibody radioimmunoassay method to measure insulin in the presence of PEG, as was suggested by Nakagawa et al.[9]

Because the serum proinsulin concentration is much lower than that of C-peptide and many C-peptide antisera react less well with proinsulin than with C-peptide, the contribution of proinsulin to serum C-peptide immunoreactivity is very small under most circumstances.[2] However, in insulin-treated diabetic patients, antibody-bound proinsulin may accumulate in the circulation and interfere with the accurate measurement of serum C-peptide.[3,7,8] We thus considered whether the PEG precipitation method could also be used to remove antibody-bound proinsulin from serum and permit the measurement of C-peptide in the supernatant. Addition of small volumes of PEG supernatant did not interfere with the C-peptide radioimmunoassay, and the C-peptide values in normal sera determined by direct assay and following PEG precipitation were in good agreement. Satisfactory recoveries of ^{125}I-tyrosylated C-peptide and unlabeled human C-peptide were also obtained. Furthermore, in experiments with sera containing insulin antibodies, the C-peptide concentrations were comparable to values determined by gel filtration. Preincubation of sera at 37° C. is obviously unnecessary for the measurement of C-peptide, because this peptide is not bound to antibodies in the circulation.

It should be noted that a small amount of free proinsulin, in equilibrium with that bound to insulin antibodies, remains in the PEG supernatant. However, as most human C-peptide antisera react less well with proinsulin than C-peptide and the free proinsulin concentration in serum is usually considerably lower than that of C-peptide, the contribution of this proinsulin to the C-peptide level will be relatively minor under most circumstances. A similar situation exists with respect to the measurement of free insulin, because the free proinsulin will also react in the insulin assay. When large amounts of C-peptide antisera become available, alternative approaches to this problem may be possible. For instance, serum C-peptide and free and insulin-antibody-bound proinsulin could presumably be removed from serum by passage over Sepharose to which human C-peptide antibodies had been coupled. The eluate could then be treated with PEG before and after acidification for the measurement of free and total insulin. However, even this method may not be as specific as one might anticipate, because of the development of bovine or porcine proinsulin connecting-segment-directed antibodies in some diabetic patients. Bovine or porcine proinsulin, present in varying amounts in some but not all[20,21] commercially available insulin preparations, may stimulate the production of these antibodies and subsequently bind to them in the circulation[22-25] (figure 9).

The development of circulating insulin antibodies within a few weeks of beginning insulin therapy in insulin-requiring diabetic patients has hindered investigations concerning the relationship of the dose, timing, and type of exogenous insulin required to control the blood sugar, the significance and quantitation of residual beta-cell function, and the biologic efficacy of insulin in these patients. These difficulties may be

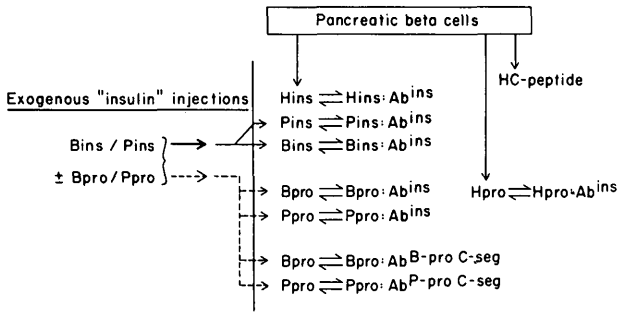

FIG. 9. Circulating antibodies in insulin-treated diabetics. Both endogenous and exogenous insulin and proinsulin are bound to insulin antibodies (Ab^{ins}). Antibodies directed to bovine or porcine proinsulin-connecting segment ($Ab_{B\text{-}pro\ C\ seg}$, $Ab_{P\text{-}pro\ C\ seg}$) bind the homologous species of proinsulin specifically. Endogenous human C-peptide is not bound to any of these antibodies. It should be noted that insulin antibodies may be more specific for bovine or porcine insulin, but this distinction is not made in the figure. H: human, B: bovine, P: porcine, ins: insulin, pro: proinsulin, pro C-seg: proinsulin-connecting segment.

overcome to a large extent by using the simple and rapid PEG precipitation technique in conjunction with the insulin and C-peptide assays. The determination of serum free insulin levels provides information about the insulin concentration, from both endogenous and exogenous sources, that is available for interacting with its receptors. Likewise, precipitation of antibody-bound proinsulin with PEG prior to assay allows serum C-peptide to be used as an accurate indicator of pancreatic beta-cell function in insulin-requiring diabetics. Combined measurements of serum free insulin and C-peptide will undoubtedly prove useful in assessing the course, prognosis, and therapy of insulin-requiring diabetics.

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