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Identification of Proinsulin and C-Peptide in Human Serum by a Specific Immunoassay*

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Abstract. An immunoassay for the measurement of human proinsulin and C-peptide is described. By a combination of this system and an insulin immunoassay, proinsulin, insulin, and C-peptide were identified in acid-ethanol extracts of serum after gel filtration. In keeping with the results of biosynthetic studies, insulin and C-peptide were found to be present in equimolar amounts. The absolute values of serum proinsulin determined independently in the two assays corresponded closely.

Proinsulin, the higher molecular weight precursor of insulin, is a single polypeptide chain of 81–86 residues ordered: NH_2-(B chain)-Arg-Arg-(C-peptide)-Lys-Arg-(A chain).[1–3] During the biosynthesis of insulin in the beta cells of the pancreas, the single-chain form first facilitates the correct laying down of the intrachain disulfide bonds of insulin and is then cleaved proteolytically to yield insulin and the C-peptide.[4–6] The C-peptide remains in the islet cells along with insulin, possibly within secretory granules, and both are subsequently liberated into the circulation.[7] It has thus been possible to infer the amino acid sequence of human proinsulin from the structure of the C-peptide isolated from human pancreas obtained at postmortem examination.[8,9]

A comparison of the amino acid sequences of the human C-peptide[9] with the sequences of the porcine[2] and bovine[6] peptides (Fig. 1) shows much greater

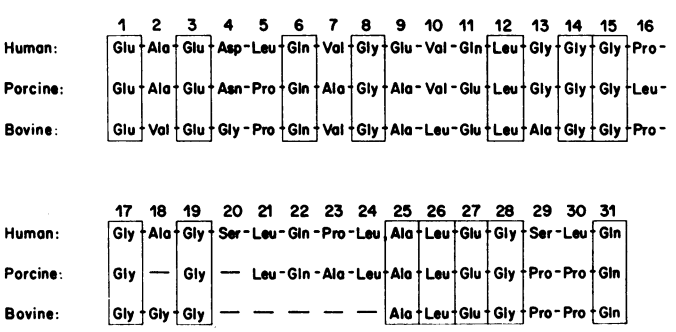

FIG. 1. Comparison of the primary structures of human, porcine and bovine proinsulin C-peptides. The enclosed residues, which are identical in all 3 species, comprise about 50% of the amino acids. Because of the differences in length of the C-peptides in these species, deletions were arbitrarily placed as shown in order to maximize homology.

variations in primary structure than occur in the insulins of these three species. Immunological comparisons of porcine, bovine, and human proinsulins with antisera to bovine and porcine proinsulins have shown very little cross-reaction after suppression or separation of the antibody molecules directed against the common immunologic determinants associated with their insulin regions.[10] Thus, to detect human proinsulin specifically in plasma with precision, it has become necessary to develop an immunoassay system directed against its C-peptide determinants. By linking the purified human pancreatic C-peptide with rabbit albumin it has been possible to produce antibodies in guinea pigs that react strongly and specifically with human proinsulin and the human C-peptide. This paper describes the immunoassay procedure and the results obtained in several normal and pathological human serum samples.

Materials and Methods. Human proinsulin was isolated from crystalline human insulin as described elsewhere.[11] Human insulin was kindly supplied by the Novo Company (Copenhagen).

Isolation of human C-peptide: Nondiabetic human pancreases obtained at necropsy were extracted in 500-g lots with acid ethanol by a modification of the procedure of Davoren.[12] After partial purification and precipitation with organic solvents, gel filtration of the extracted protein was carried out on an 8 × 100 cm column of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, Calif.) eluted with 3 M acetic acid. The fractions containing both insulin and C-peptide were combined and lyophilized. After separation of insulin and glucagon by passage of the protein over a column of carboxymethyl cellulose (CM-23) at pH 5.0 (0.01 M Na citrate, 7 M urea) the acidic peptide fraction containing the C-peptide was further purified by paper electrophoresis and partition chromatography as described in detail elsewhere.[8,14] Purity (>98%) was assessed in several systems and by amino acid analysis (Beckman-Spinco, model 120C). Only N-terminal glutamic acid was found by dansylation.[13] The amino acid sequence of the 31 residue C-peptide was determined by Edman degradation of the whole peptide and of derived chymotryptic and thermolytic peptides.[9,14] Monkey C-peptide was isolated from pancreas of *Cercopithecus aetheops* in the same manner.

Immunization: Human C-peptide, 3 mg, was covalently attached to 1.5 mg of rabbit serum albumin using 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (400 mg/ml).[15] A tracer of [^{131}I]tyrosine C-peptide (see below) was added to the mixture and evidence for the successful conjugation of the reactants to albumin was provided by the change in elution position of the labeled material after gel filtration on a Bio-Gel P-30 column. After separation of the carbodiimide by gel filtration on Sephadex G-10 the conjugate was dissolved in 1 ml of 0.9% saline, suspended in 3 ml of complete Freund's adjuvant and injected into the foot pads and multiple subcutaneous sites of two guinea pigs (each immunization dose contained approximately 0.35 mg of C-peptide and 0.2 mg of rabbit albumin). Three subsequent immunizations were given at 2–3 week intervals and the animals were bled 10 days after the third and fourth injection.

Preparation of tyrosylated human C-peptide: Human C-peptide (0.5 mg) was dissolved in 0.15 ml of 0.0025 N hydrochloric acid, and 0.15 ml of 0.1 M sodium phosphate buffer (pH 7.6) was added. 2 mg of *N*-carboxytyrosyl anhydride (Cyclo Chemical Corp., Los Angeles) was dissolved in 0.1 ml of anhydrous dioxane and added to the C-peptide in an ice bath at 2°C.[16] The mixture was allowed to react for 16 hr with continual stirring. The small precipitate which formed was removed by centrifugation and the supernant was diluted in 0.8 ml of 3 M acetic acid and applied to a 1 × 50 cm Bio-Gel P-30 column. The fractions containing the tyrosylated C-peptide were identified by the ninhydrin reaction and combined and concentrated under reduced pressure. Thereafter, Veronal buffer, pH 8.6, ionic strength 0.075 was added to give a peptide concentration of 100 μg/ml.

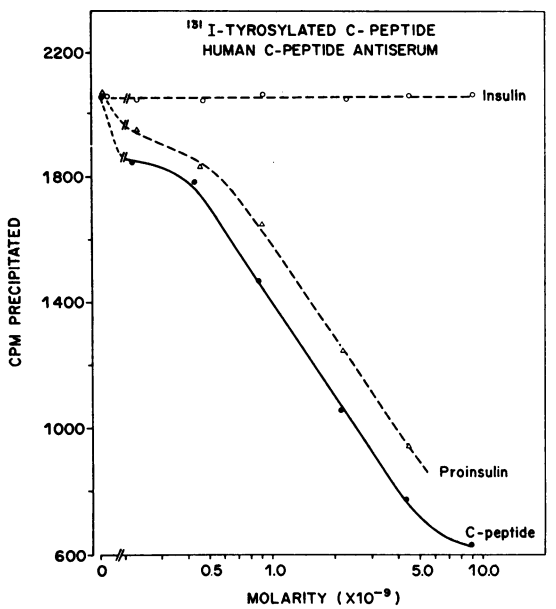

FIG. 2. Immunoassay of human C-peptide, proinsulin, and insulin using human C-peptide antiserum and [^{131}I] tyrosylated human C-peptide. Porcine and bovine C-peptides, proinsulin, and insulin did not react in this system.

Iodination: 5-μg samples of tyrosylated C-peptide were iodinated with 3 mCi of ^{131}I (carrier-free; Union Carbide Corp., N.Y.) by the method of Hunter and Greenwood.[17] The [^{131}I]Tyr-C-peptide was purified by gel filtration on Bio-Gel P-30 in 3 M acetic acid. The 50 $\times$ 1 cm column and collecting tubes were first coated with bovine albumin (Pentex Corp., Ill) in 50% acetic acid (10 mg/ml) to minimize adsorption onto glassware. The peaks of radioactivity were identified by counting an aliquot of each fraction. The iodinated tyrosylated C-peptide eluted slightly later from the column than did the unmodified peptide, and fractions comprising the ascending limb of its peak were combined for use in the immunoassay.

Immunoassay: A modification of the method of Morgan and Lazarow[18] was used. The buffer was 0.1 M Tris·HCl, pH 7.7, containing 0.05 M sodium chloride and 0.25% bovine albumin. The volume of the first reaction mixture was 1.0 ml and the incubation time 48–72 hr. The antigen–antibody complex was precipitated by adding 0.1 ml of rabbit antiserum to guinea pig globulin (Sylvana Co., N.J.). After centrifugation at 4°C for 20 min at 2000 rpm, the supernatant fluid was decanted and the precipitate counted in an automatic gamma-spectrometer.

Two assay systems were used: human insulin and proinsulin standards, [^{131}I]porcine insulin and porcine insulin antiserum; and human C-peptide and proinsulin standards, tyrosylated [^{131}I]C-peptide, and the C-peptide antiserum.

Fractionation of serum: Aliquots of serum (1.5–10 ml) were extracted with acid ethanol as described previously.[19] The extracts were fractionated on 1 $\times$ 50 cm columns of Bio-Gel P-30, 100–200 mesh, equilibrated in 3 M acetic acid, and calibrated with porcine [^{131}I]insulin and [^{131}I]proinsulin. The fraction size was 1.2 ml and the collecting tubes were coated with Siliclad (Clay-Adams, Inc., N.Y.). The fractions were evaporated to dryness under reduced pressure and after addition of the albumin-containing buffer described above, aliquots were taken for immunoassay

Patients: Serum samples were obtained, from fasted subjects and 60 min after the oral administration of 100 g of glucose, from two young healthy male subjects of normal weight and a markedly obese (480 lb) 16-yr-old girl In addition, sera from two fasting patients, each with an islet cell carcinoma or adenoma, were studied.

Results. Human and monkey C-peptide and human proinsulin competed with tyrosylated human [^{131}I]C-peptide for sites in the C-peptide antiserum (Fig. 2). In contrast, porcine and bovine insulin, proinsulin, and C-peptide (besides

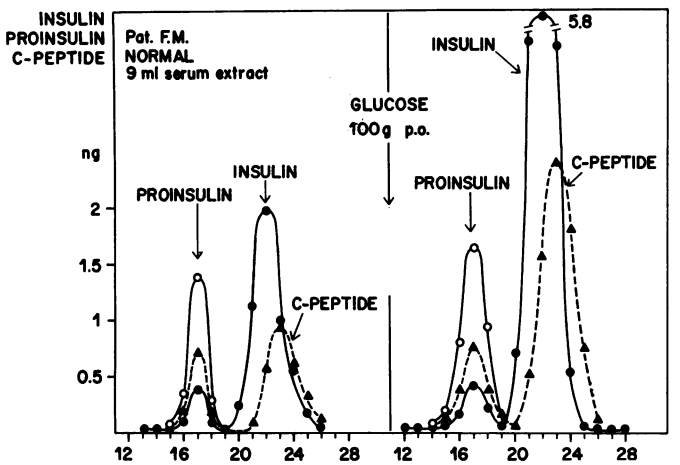

FIG. 3. Serum was taken from a healthy fasted subject, and 60 min after 100 g of glucose was administered orally to the same subject. After acid-ethanol extraction and gel filtration on a Bio-Gel P-30 column, the fractions were assayed as follows:
(1) Insulin assay: insulin standard ●—●; proinsulin standard O—O.
(2) C-peptide assay: C-peptide standard ▲- - -▲.

human insulin) did not react in this system. The sensitivity of the immunoassay lay between 0.05 and 0.1 ng/ml, with a final antiserum dilution of 1:1000, and tracer with specific activity of 50 mCi/mg. Human proinsulin reacted approximately two-thirds as well as the C-peptide when expressed on a molar basis.

After gel filtration, immunoreactive material was detected in fractions corresponding to proinsulin and insulin marker peaks using the insulin assay system (Figs. 3 and 4). As the proinsulin reacted less efficiently than insulin on both a

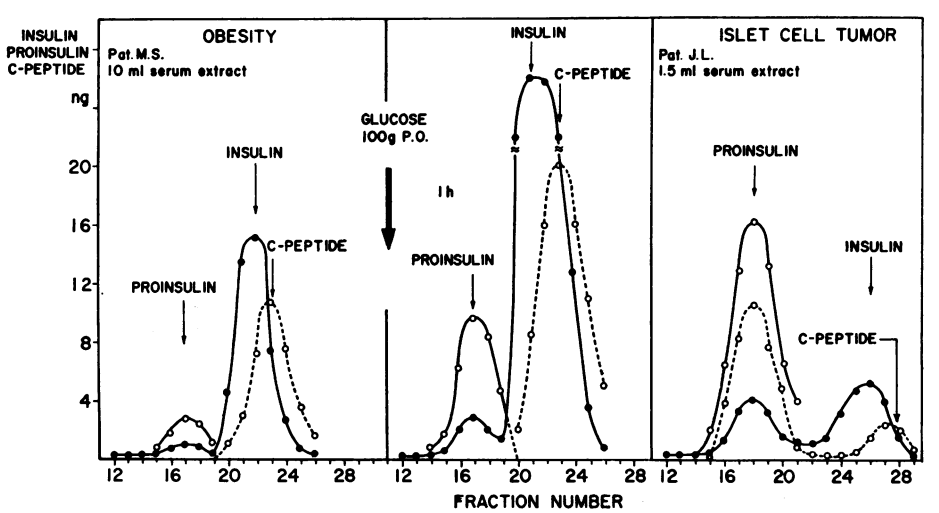

FIG. 4. Serum was taken from an obese fasted subject and 60 min after 100 g of glucose was administered orally in the same subject (left two panels) and from a fasted patient with an islet cell carcinoma (*right panel*). After acid-ethanol extraction and gel filtration on a Bio-Gel P-30 column, the fractions were assayed as follows:
(1) Insulin assay: insulin standard ●—●; proinsulin standard O—O.
(2) C-peptide assay: C-peptide standard O- - -O.

TABLE 1.

Sample	Insulin ng/ml	Insulin fmol/ml	C-Peptide ng/ml	C-Peptide fmol/ml
1	0.58	99	0.28	93
2	1.6	275	0.8	264
3	4.8	827	2.9	960
4	29.5	5080	8.5	2810
5	1.2	206	0.65	215
6	5.8	999	2.8	927
7	1.4	241	0.75	248
8	13.4	2310	6.6	2180

Insulin and C-peptide content of acid–ethanol extracts of serum samples after gel filtration on Bio-Gel P-30. The insulin values represented the sum of the individual fractions of the insulin peak as measured with the insulin standard in the insulin immunoassay. The C-peptide concentrations were determined similarly, using the C-peptide standard in the human C-peptide assay. fmol, femtomoles (10^{-15} mole).

weight and a molar basis, the absolute values of proinsulin (early eluting peak) measured by this standard were 3 to 4 times higher than the levels obtained from the insulin standard. Both proinsulin and insulin showed a rise at 60 min after the administration of glucose in the normal and obese subjects.[19] The levels of proinsulin in the patients with islet cell tumors were markedly increased and comprised 60–80% of the total immunologic activity.

Assay of the fractions with the C-peptide immunoassay (Figs. 3 and 4) also showed two peaks in every sample tested (since the proinsulin from obese subjects had been used for other tests,[19] there was insufficient material for this experiment). The early peak corresponded to that of proinsulin and the later to that of marker C-peptide, which eluted 1–2 tubes after the insulin. After glucose administration, the levels of C-peptide increased markedly from fasting values. The absolute concentrations of C-peptide in the patients with islet cell tumors were relatively low in comparison to their elevated proinsulin levels. When the concentrations of insulin and C-peptide were evaluated on a molar basis, essentially equimolar amounts of each were present in all the samples (Table 1). Furthermore, the absolute levels of proinsulin read from the proinsulin standard in the C-peptide assay corresponded closely to the values obtained using the same standard in the insulin assay (Table 2).

TABLE 2.

Sample	(A) Insulin assay (ng/ml)	(B) C-Peptide assay (ng/ml)
1	4.6	4.8
2	2.1	1.8
3	9.8	10.0
4	1.0	1.0
5	3.0	3.2
6	4.8	4.6
7	1.8	1.6
8	5.6	5.4

Proinsulin values of acid-ethanol extracts of serum samples after gel filtration on Bio-Gel P-30. The concentrations were estimated from the proinsulin standard in (*A*) the insulin immunoassay system (porcine insulin antiserum, [^{131}I]porcine insulin) and (*B*) the C-peptide immunoassay system (human C-peptide antiserum, [^{131}I]tyrosylated human C-peptide).

Discussion. Although bovine and porcine proinsulin are excellent antigens in guinea pigs, repeated immunization with C-peptide alone did not result in the production of antibodies. Similar difficulties with proteins and polypeptides of low molecular weight such as secretin, glucagon, gastrin, angiotensin, and bradykinin have been encountered by others. Under these circumstances, coupling of the antigen to a larger protein such as rabbit albu-

min has often led to the formation of antibodies that are satisfactory for use in the immunoassay methods.[15] In contrast to other conjugating procedures, the reaction with carbodiimide has the advantage of occurring under mild conditions and of conjugating haptens directly to proteins without interposing additional groups between the hapten and carrier. Despite the relatively small quantities of purified human C-peptide available for immunization, coupling to albumin did yield useful antibodies after three injections containing a total of 1.0 mg of C-peptide and 0.6 mg of rabbit albumin.

The human, porcine, and bovine C-peptides contain 26–31 amino acids, but these do not include tyrosine. Although successful iodination of secretin, which also does not contain this amino acid, has been described,[20] we chose to couple tyrosine to the C-peptide to facilitate this reaction and ensure labeled tracers of high specific activity. The reaction of many proteins with *N*-carboxyanhydride derivatives of amino acids has led to remarkably little alteration in their immunological and biological activity. Thus Anfinsen *et al.*[21] have shown that reduced polyalanyl ribonuclease, with an average of 50–100 added alanines per molecule, is capable of refolding to form the active enzyme, while Fuchs and Sela[22] demonstrated that poly-D,L-alanyl rabbit gamma globulin, with about 700 alanine residues attached to each molecule, preserves its capacity to bind antigen. Similarly, human growth hormone to which 23 alanine residues were coupled retained 95% of its biological activity.[23] This preservation of biological activity is probably due to the fact that under the gentle conditions of the reaction (2°C and neutral pH) only the exposed alpha or epsilon amino groups react with the anhydride, and the positive charges in these regions are replaced by the positive charges of the alpha amino groups of the added amino acids.

The amino acid sequence of human C-peptide differs from that of the porcine and bovine molecules by about 50%.[2,6,9] Because of these major differences in primary structure it was anticipated that antibodies against each of these molecules would be highly species-specific. This is supported by the specific reaction of porcine and bovine C-peptide with their respective proinsulin antisera and the failure of human C-peptide to react in either system.[10] The present results show that only human proinsulin and C-peptide and the closely related monkey C-peptide compete with [^{131}I]tyrosylated human C-peptide for binding to antibodies raised against human C-peptide and confirm the immunological specificity of this antiserum for antigenic determinants in human C-peptide. The reduced affinity of human proinsulin compared to C-peptide in this system is presumably due to the additional presence in proinsulin of the four basic connecting residues and the insulin moiety. These may either alter the tertiary configuration of the peptide or sterically hinder certain of its antigenic determinants.

Because of the presence of both proinsulin and C-peptide in blood, and the cross reactivity of these proteins in the human C-peptide immunoassay system, direct measurement of these proteins in unextracted serum was not possible. Stoll *et al.*[24] have produced an antiserum to porcine proinsulin which does not react significantly with either insulin or C-peptide. However, at present, sufficient quantities of human proinsulin are not available for immunization of animals and thus the absolute concentrations of proinsulin, insulin, and C-peptide

can be measured with insulin and C-peptide assays only after preliminary separation by gel filtration. The close correspondence of the absolute levels of proinsulin determined against the human proinsulin standard in these two different assay systems provides convincing evidence that the values obtained correspond to the true serum concentrations.

The finding of C-peptide in plasma in amounts corresponding to the concentration of insulin is in keeping with the results of biosynthetic studies that used isolated rat islets of Langerhans. In this system, incorporation of leucine or proline into material having the composition and general properties of C-peptide could be demonstrated.[6] Initially only proinsulin was synthesized, but as incubation proceeded both insulin and C-peptide began to accumulate.[6,7] The amount of labeled leucine incorporated corresponded to the number of leucines in each peptide indicating that the peptides were produced in equal amounts as products of the proteolytic transformation of proinsulin.[7] This equivalence was maintained over many hours of incubation of islets *in vitro* and was also found to be the case for the material secreted into the medium.[7] The secretion of these substances in a one-to-one ratio was of special interest, as it suggested that the C-peptide and insulin are packaged together in the storage granules and secreted by a process of emiocytosis[25] in which the entire granule contents are liberated at the cell surface. In keeping with this hypothesis, extracts of bovine[8] (and porcine (unpublished results)) pancreas yielded approximately equal amounts of insulin and C-peptide.

All these results are in accord with the hypothesis that the conversion of proinsulin to insulin occurs within the secretory granules or the Golgi apparatus.[6,26] Sorensen *et al.*[27] have recently presented evidence supporting this hypothesis based on the distribution of labeled proinsulin and insulin in islet subcellular fractions. Such a mechanism would account for the retention of all of the proteolytic conversion products. Although the mechanism of the transformation has not yet been elucidated, it seems to be clear that the basic residues occurring as pairs at either end of the connecting segment in proinsulin are eliminated either as dipeptides or as free amino acids. Thus, while the data reported here do not permit the conclusion that the C-peptide material in plasma is identical to the material isolated from the pancreas, it seems unlikely that carboxyl terminal basic residues are present, and the identical elution position of the plasma peptide on Bio-Gel P-30 rules out any extensive degradation of the material before or during its secretion into the plasma.

The presence of C-peptide and insulin in equimolar concentrations in both the pancreas and peripheral circulation suggests that the hepatic extraction of these two proteins might be similar. However, as yet, definitive experiments concerning the space of distribution, metabolic clearance rate, and hepatic and renal metabolism of the C-peptide have not been done. Preliminary studies have shown no effect of the C-peptide on several parameters of insulin action *in vitro*.[28] Nevertheless, its potential value as an independent indicator of beta cell function under various metabolic conditions is significant. In particular, the use of this assay in insulin-requiring diabetic patients with circulating insulin antibodies may provide, for the first time, a direct and convenient measurement of residual beta cell function.

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