# Measurement of Circulating Human Proinsulin Concentrations Using a Proinsulin-specific Antiserum

C. F. DEACON AND J. M. CONLON

**SUMMARY**

**Antibodies have been raised against biosynthetic human proinsulin that show <1% cross-reactivity with human insulin and C-peptide. A sensitive ($IC_{50}$ 0.16 pmol/ml; minimum detectable concentration 0.004 pmol/ml) radioimmunoassay has been developed using this antiserum and $^{125}$I-proinsulin that will measure proinsulin-like immunoreactivity in human serum without the need for prior separation of insulin or C-peptide. In healthy, fasted subjects (N = 23), the serum proinsulin concentration was 0.015 ± 0.001 pmol/ml (mean ± SEM). In six healthy subjects, serum proinsulin rose to 250% of basal after 120 min in response to 100 g oral carbohydrate, but to only 130% after 60 min following 25 g oral carbohydrate. The proinsulin/total immunoreactive insulin ratio and the proinsulin/C-peptide ratio fell sharply after both high and low carbohydrate loads. Endogenous human serum proinsulin-like immunoreactivity released into the circulation after 100 g carbohydrate was eluted from a Mono Q high-performance, ion-exchange column with the same retention time as biosynthetic human proinsulin. Treatment of biosynthetic proinsulin with trypsin under mild conditions led to a decrease in proinsulin-like immunoreactivity concomitant with an increase in C-peptide and insulin-like immunoreactivity, indicating that the proinsulin-specific antiserum did not preferentially recognize intermediates of proinsulin cleavage.** **DIABETES 1985; 34:491–97.**

Assessment of the importance of proinsulin in metabolic regulation and in the pathogenesis of disease has been hampered by the lack of availability of human proinsulin. With recent advances in recombinant DNA technology, however, biosynthetic human proinsulin is now available in relatively large amounts.[1] Its biologic activity in vitro has recently been investigated.[2] Measurement of proinsulin-like immunoreactivity in blood by radioimmunoassay is complicated by the fact that the molecule contains the antigenic determinants of both insulin and C-peptide. Thus, total immunoreactive insulin (IRI), measured with an antiserum raised against insulin, represents the sum of contributions from insulin and proinsulin and, similarly, proinsulin will cross-react with most antibodies raised against C-peptide. Previous attempts to overcome this problem have employed gel filtration to separate immunoreactive components before assay[3-5] or have used an "insulin-specific protease" to selectively degrade insulin.[6] More recently, in a two-step assay involving the binding of proinsulin to immobilized antibodies against insulin and removal of free C-peptide, proinsulin is quantitated using a C-peptide antibody.[7] These procedures are tedious to perform and results may be irreproducible in different laboratories.

In this study, we describe the production and use of an antiserum to biosynthetic human proinsulin that does not cross-react with insulin and C-peptide. This antibody enables direct measurement of proinsulin-like immunoreactivity in human serum in one incubation step without the need for prior separation of insulin and C-peptide. The radioimmunoassay has been used to compare the release of beta cell peptides in response to a high (100 g) and to a low (25 g) oral carbohydrate load in healthy subjects.

## MATERIALS AND METHODS

Human proinsulin (lot no. 581-5H7-24A) was kindly supplied by Eli Lilly and Company, Indianapolis, Indiana. Human C-peptide was purchased from Bachem Feinchemikalien AG, Switzerland. Human insulin and $^{125}$I-[Tyrosine-1]C-peptide were from Novo Research Institute, Denmark. Materials for chromatography were from Pharmacia, Sweden. L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was from Sigma Chemical Company, St. Louis, Missouri.

**Preparation of antibodies to proinsulin.** Male albino guinea pigs (300–400 g, N = 6) were injected at multiple subcu-

From the Clinical Research Group for Gastrointestinal Endocrinology, University of Göttingen, Max-Planck-Gesellschaft, Goslerstrasse 10d, D-3400 Göttingen, FRG.
Address reprint requests to J. M. Conlon at the above address.
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taneous sites with an emulsion (2 ml/animal) formed from equal volumes of Freund's adjuvant (complete for the primary immunization and incomplete for subsequent injections) and 0.04 M sodium phosphate buffer, pH 7.4, containing proinsulin (40 μg).[8] Animals were boosted at 3-wk intervals and blood (2 ml) was taken by cardiac puncture 2 wk after the injection. After clotting (6 h at room temperature), serum was stored at −70°C.

**Preparation of $^{125}$I-labeled proinsulin.** Iodination was carried out using a modification of the iodination procedure of Fraker and Speck.[9] Proinsulin (5 μg in 100 μl of 0.05 M sodium phosphate buffer, pH 7.4) was incubated with Na$^{125}$I (10 μl, 1 mCi) for 5 min at 4°C in a tube containing a film of iodogen (15 μg). The reaction mixture was chromatographed on a column (30 cm × 0.9 cm) of QAE-Sephadex A-25 equilibrated with 0.08 M Tris-HCl, 0.08 M NaCl adjusted to pH 8.6 with 0.02 M HCl and containing human serum albumin (10 mg/ml). The column was eluted at 4°C and at a flow rate of 15 ml/h with a linear gradient formed from starting buffer (150 ml) and starting buffer containing 0.3 M NaCl (150 ml). Fractions (2.5 ml) were collected and radioactivity counted in an LKB 1274 gamma counter (efficiency 75%).

**Radioimmunoassay procedures.** Proinsulin antiserum (100 μl, final dilution 1:24,000) and proinsulin standards (100 μl, 0.004–1.0 pmol/ml) were incubated at 4°C for 72 h in 0.04 M sodium phosphate buffer, pH 7.4, containing 0.1% human serum albumin and 0.02% thiomersal (final volume 300 μl). $^{125}$I-proinsulin (100 μl, approximately 10,000 cpm) was added, and after a further 24-h incubation, antibody-bound radioactivity was precipitated by the addition of bovine γ-globulin (100 μl, 10 mg/ml) and polyethylene glycol 6000 (1 ml, 20% wt/vol). The specificity of the antiserum was determined by incubations in the presence of human insulin, human C-peptide, glucagon, somatostatin, substance P, and VIP.

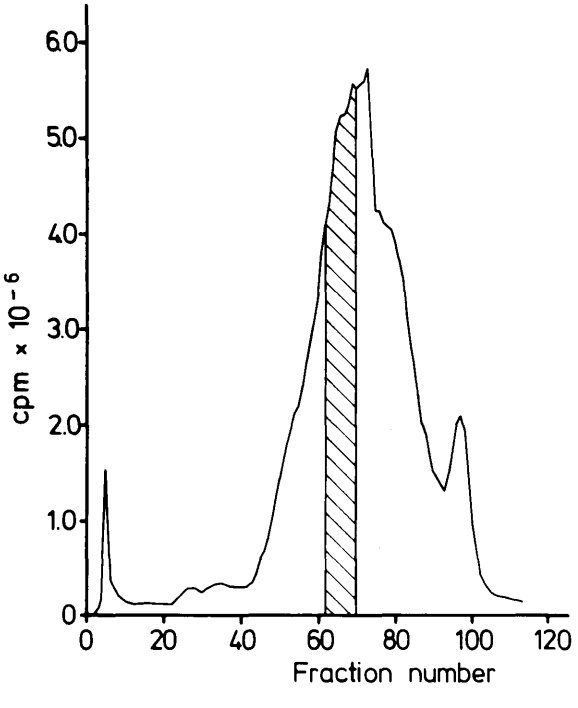


**FIGURE 1. Purification of radioiodinated biosynthetic human proinsulin: elution profile on QAE-Sephadex-A 25. The fractions represented by the hatched area (sp. act. 410 Ci/mmol) showed maximum binding to an excess of proinsulin antiserum and were used in radioimmunoassay.**

TABLE 1
Specificities of antisera raised against biosynthetic human proinsulin

| Guinea pig | % Cross-reactivity toward $^{125}$I-[Tyr-1]C-peptide | % Cross-reactivity toward $^{125}$I-insulin |
|---|---|---|
| 1 | 41.1 | 0.4 |
| 2 | 0 | 91.8 |
| 3 | 9.3 | 73.5 |
| 4 | 0 | 1.3 |
| 5 | 1.2 | 28.2 |
| 6 | 1.0 | 28.5 |

Sera from animal 4 was used in radioimmunoassay. Antisera were tested at a dilution of 1:24,000. Reactivity with [$^{125}$I]proinsulin is defined as 100%. Antisera were incubated with approximately equal numbers of counts per minute of each tracer.

Immunoreactive C-peptide was assayed according to the method of Heding[8] using antibody M 1230 (Novo Research Institute) except that polyethylene glycol 6000 was used to separate antibody-bound radioactivity. Immunoreactive insulin was measured using an anti-porcine insulin serum raised in guinea pigs with human insulin standards.[10] Serum glucose was determined by the glucose-oxidase method using a Beckman glucose analyzer.

**Response to oral carbohydrate in healthy subjects.** All subjects studied were within 15% of ideal body weight and were free from known endocrine, gastrointestinal, and metabolic disease. After a fast of at least 12 h, a single blood sample (10 ml) was taken via an antecubital vein from 23 subjects (11 male, mean age 28 ± 1 yr). After an overnight fast, an indwelling cannula was placed in an antecubital vein of six subjects (4 male, mean age 30 ± 2 yr). Two basal samples (10 ml) were taken at 10-min intervals and the subjects received, by mouth over 5 min, a carbohydrate load equivalent to 100 g glucose in 400 ml water (Boehringer Dextro OGT). Blood samples (10 ml) were taken at 30-min intervals for 240 min. After an interval of 2 mo, the same six fasting subjects received oral carbohydrate equivalent to 25 g glucose in 400 ml water. Samples were collected for only 180 min, as a pilot experiment had indicated that all parameters had returned to basal values by this time.

**Sample preparation.** After clotting and centrifugation at 4°C (1600 × *g* for 10 min), high-molecular-weight components were removed from sera (1 ml) by addition of ethanol (1.6 vol). After centrifugation (1600 × *g* for 30 min), the supernatants were evaporated to dryness using a Savant speed-vac concentrator. The residue was redissolved in assay buffer resulting in a twofold increase in concentration. In order that the tubes of the standard curve should contain an equivalent amount of serum extract, pooled human serum was made proinsulin-free by treatment with charcoal before ethanol precipitation.[11] Curves were set up using this extract at the appropriate concentration.

**Characterization of human proinsulin-like immunoreactivity using high-performance, ion-exchange chromatography.** A mixture of human proinsulin (10 μg), human insulin (10 μg), and human C-peptide (10 μg) was chromatographed on a Mono Q FPLC column equilibrated with 0.05

M Tris-HCL, pH 8.9, containing 60% (vol/vol) ethanol. The column was eluted over 20 min at a flow rate of 0.8 ml/min with a linear gradient formed from starting buffer and starting buffer containing 105 mM NaCl. The column effluent was monitored at 214 nm and fractions (20 s) were assayed at appropriate dilution for immunoreactive proinsulin, insulin, and C-peptide. Pooled, deproteinized serum (15 ml, taken 120 min after the 100-g oral carbohydrate) was extracted using a Sep-Pak C18 cartridge as previously described.[12] The material recovered from the cartridge was dissolved in starting buffer (500 μl) and chromatographed under the previous conditions.

**Limited trypsinization of proinsulin.** Biosynthetic human proinsulin (57 μg) was incubated with TPCK-treated trypsin (0.05 μg) in 100 mM Tris-HCl buffer, pH 7.5 (100 μl), at room temperature for (1) 3 min and (2) 4 min. Reaction was stopped by addition of phenylmethylsulfonylfluoride (2 μl of a 100-mM solution) and proinsulin-like, C-peptide-like, and insulin-like immunoreactivities were measured at appropriate dilutions.

**Statistical analysis.** Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was calculated using Student's *t*-test for paired or unpaired data as appropriate. Hormone concentrations were calculated using an LKB Spline function program.

## RESULTS

**Preparation of $^{125}$I-labeled proinsulin.** The elution profile of the reaction mixture from the radioiodination of proinsulin is shown in Figure 1. Approximately 89% of the radioactivity in the fractions indicated by the hatched area bound to an excess of proinsulin antiserum (1:600 dilution). The specific activity of the label, determined as described,[13] was 45 μCi/μg. Human proinsulin contains four tyrosine residues and it is probable that label purified by this procedure represents a mixture of several components. The preparation and purification of [$^{125}$I-Tyr$^{A14}$]proinsulin by HPLC has been described,[2] but this method has the disadvantage that the apparatus becomes highly contaminated with radioactivity.

**Characterization of antibodies to proinsulin.** Within 3 mo, all animals produced antibodies to proinsulin of sufficiently high titer (>1:10,000 dilution) to be of use in radioimmunoassay. This indicates that human proinsulin is strongly immunogenic in the guinea pig. The specificities of the antisera are compared in Table 1. Under radioimmunoassay conditions, serum from animal 4 showed no detectable binding of $^{125}$I-[Tyrosine-1]human C-peptide and binding of $^{125}$I-insulin was only 1.3% of that of an equivalent amount of $^{125}$I-proinsulin. This antiserum (Go 4) was used at a final dilution of 1:24,000 in subsequent assays.

The effect of increasing concentrations of proinsulin, insulin, and C-peptide on the binding of $^{125}$I-proinsulin to antiserum Go 4 is shown in Figure 2. Human C-peptide and insulin showed very low cross-reactivity toward the antiserum. At a C-peptide concentration of 10 pmol/ml, binding of $^{125}$I-proinsulin was suppressed by 5.7%, which corresponds to a proinsulin concentration of 0.01 pmol/ml (i.e., a cross-reactivity of 0.1% on a molar basis). At an insulin concentration of 500 mU/L, no inhibition of binding of $^{125}$I-proinsulin was detected. Similarly, no inhibition of binding by glucagon, somatostatin, VIP, and substance P at concentration of 10 pmol/ml was detected.

The mid-range ($IC_{50}$) of the assay was 0.16 pmol/ml and the minimum detectable concentration (causing a fall of 2 SD from $B_0$) was 0.008 pmol/ml, which is equivalent to a minimum concentration of 0.004 pmol/ml in serum before concentration. The discriminatory sensitivity in the physiologic range (0.007–0.042 pmol/ml for healthy subjects) was 0.002 pmol/ml. The intra- and interassay coefficients of variation were 4.1% and 8.1% for 0.032 pmol/ml proinsulin standard, respectively. The recoveries of proinsulin (0.1 pmol) added to serum before the ethanol precipitation procedure were in the range 91–114% (N = 9). The levels of proinsulin-like immunoreactivity in the serum of healthy subjects are too low to demonstrate that the endogenous immunoreactivity dilutes in parallel with the standard. However, serial dilutions of the proinsulin-like immunoreactivity in the serum of a patient with an insulinoma (1.16 pmol/ml) resulted in a line with a slope parallel to that of the proinsulin standard when incubated under the usual radioimmunoassay conditions.

The ability of proinsulin to inhibit the binding of $^{125}$I-[Tyrosine-1]C-peptide to an antibody to C-peptide and to inhibit the binding of $^{125}$I-insulin to an antibody to insulin is shown in Figure 3. The cross-reactivity of proinsulin in the insulin assay is comparable to the value reported by Madsbad et al.[14] (66% of the proinsulin concentration), but proinsulin

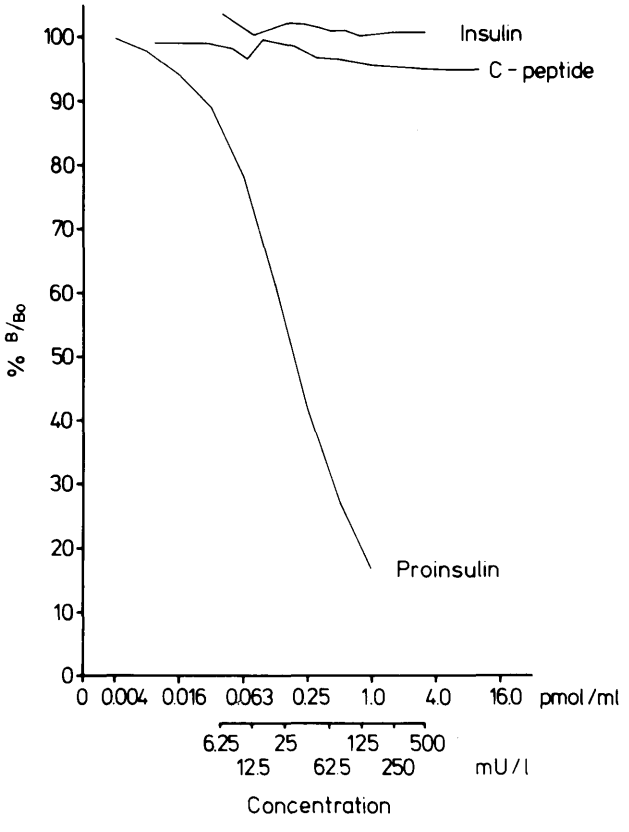


**FIGURE 2. Effect of proinsulin, C-peptide, and insulin on the binding of $^{125}$I-proinsulin to antibody Go 4 raised against human proinsulin. Proinsulin and C-peptide concentrations are expressed as pmol/ml and insulin concentration as mU/L. $B_0$ refers to binding of label in the absence of unlabeled peptides and represents 30–40% of the total radioactivity.**

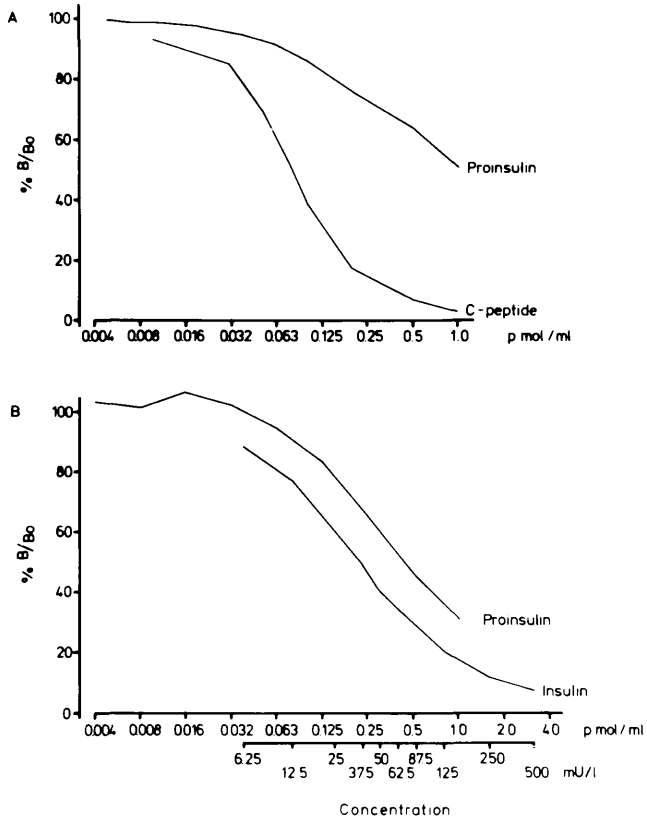


**FIGURE 3. Effect of proinsulin and C-peptide on the binding of $^{125}$I-[Tyrosine-1]C-peptide to an antibody raised against C-peptide (panel A) and the effect of proinsulin and insulin on the binding of $^{125}$I-insulin to an antibody raised against insulin (panel B).**

shows nonparallel dilution in the C-peptide assay. It can be seen, however, that interference by proinsulin in the C-peptide assay is not negligible.

**Response of serum proinsulin, C-peptide, and IRI to oral carbohydrate.** In 23 fasting, healthy subjects, the concentration of serum proinsulin-like immunoreactivity was 0.015 ± 0.001 pmol/ml (range 0.007–0.024 pmol/ml), C-peptide 0.46 ± 0.03 pmol/ml, IRI 9.91 ± 1.01 mU/L, and glucose 4.9 ± 0.1 mmol/L. The response of these parameters to high (100 g) and low (25 g) oral carbohydrate loads in six healthy subjects is shown in Figures 4 and 5. After high carbohydrate, successive rises to maximum concentrations of serum glucose (30 min), IRI (60 min), C-peptide (90 min), and proinsulin (120 min) were observed. Serum proinsulin rose approximately 250% and remained significantly elevated for 210 min. The response of IRI and C-peptide to low carbohydrate was similar except that peak concentrations were reached earlier (30 min). The response of proinsulin was more rapid with a maximum rise of 30% over mean basal values occurring after 30–60 min. Circulating proinsulin levels had returned to basal values after 150 min.

In the fasted states, proinsulin-like immunoreactivity constituted 31 ± 4% of the total IRI and 3.6 ± 0.3% of C-peptide on a molar basis (N = 23). As shown in Figure 6, the molar ratios of proinsulin to IRI and of proinsulin to C-peptide fell sharply after carbohydrate. After the high load, the ratios remained significantly depressed over 240 min but, after low carbohydrate, they returned to basal values after 90 min.

**Partial characterization of serum proinsulin-like immunoreactivity by ion-exchange chromatography.** As shown in Figure 7, proinsulin (retention time 12.0 min), insulin (13.7 min), and C-peptide (5.3 min) were completely resolved, under the conditions of chromatography, on a Mono Q ion-exchange column. Analysis of the effluent fractions by radioimmunoassay showed that only one peak of immunoreactivity was detected using the antibody to proinsulin (panel C) but two peaks were detected using antibody to C-peptide (panel D) and using the antibody to insulin (panel E). This result provides a validation of the specificity of the radioimmunoassay. As shown in panel A, endogenous serum proinsulin was eluted from the column as a single peak with the same retention time as biosynthetic human proinsulin together with broad shoulders before and after the peak.

**Effect of limited trypsinization on proinsulin-like immunoreactivity.** Treatment of biosynthetic proinsulin with a low concentration of trypsin for 3 min resulted in a decrease in

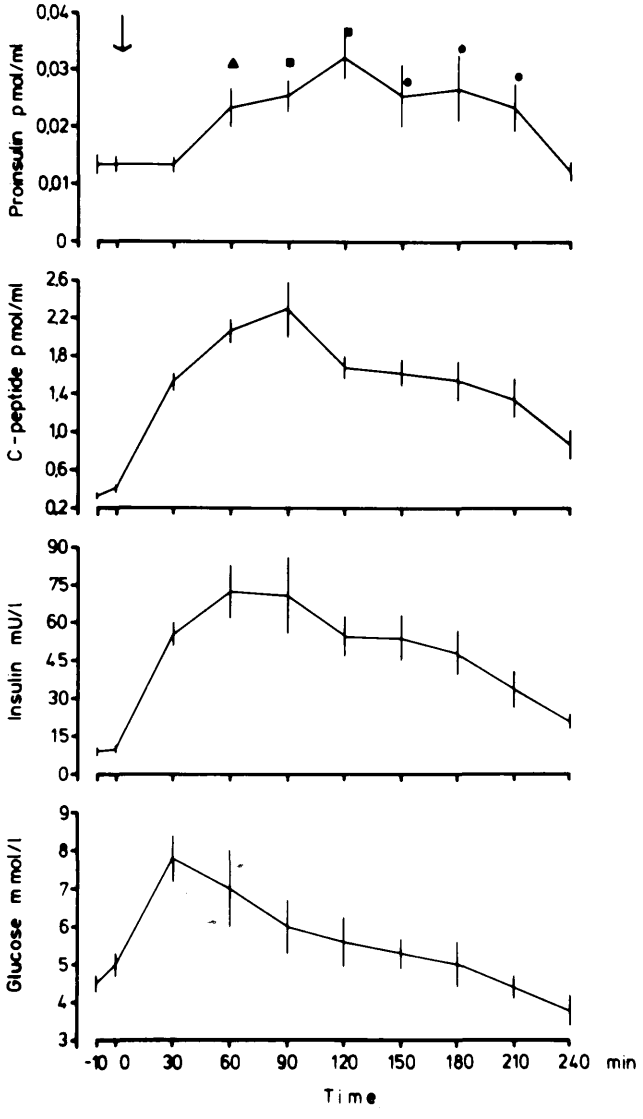


**FIGURE 4. Response of immunoreactive proinsulin, immunoreactive C-peptide, immunoreactive insulin, and serum glucose to oral carbohydrate equivalent to 100 g glucose in healthy subjects (N = 6). Significant rises over basal values are shown only for proinsulin (■ $P < 0.01$, ▲ $P < 0.02$, and ● $P < 0.05$).**

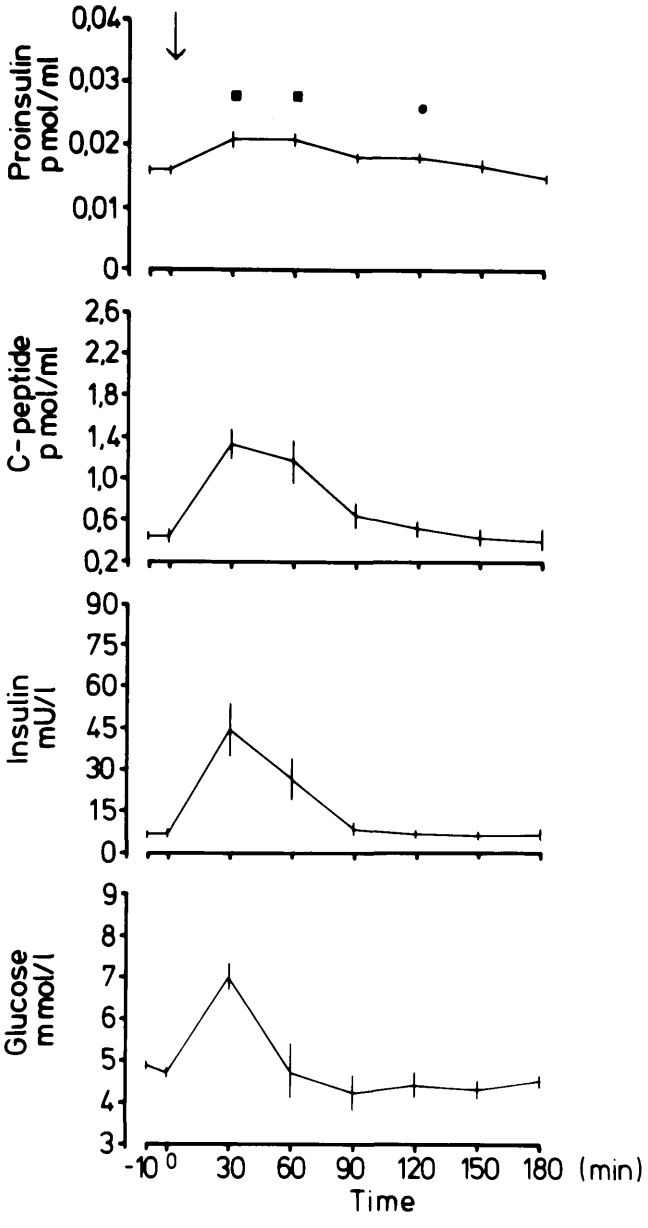


**FIGURE 5. Response of immunoreactive proinsulin, immunoreactive C-peptide, immunoreactive insulin, and serum glucose to oral carbohydrate equivalent to 25 g glucose in healthy subjects (N = 6). Symbols represent same P-values as those in Figure 4.**

proinsulin-like immunoreactivity to 12% of the initial value together with an increase in C-peptide immunoreactivity to 162% and IRI to 196%. Prolonging the reaction time to 4 min led to a fall in proinsulin-like immunoreactivity to 3% of the pretrypsinization concentration together with a rise in C-peptide immunoreactivity to 140% and IRI to 245% of initial values.

## DISCUSSION

This report describes a technically simple assay, using only one incubation step, for the measurement of proinsulin-like immunoreactivity in human serum extracts. The assay differs from previously described methodology in that it uses an antibody that is specific for proinsulin and is without appreciable reactivity toward insulin and C-peptide. Previous studies using gel filtration to partially purify proinsulin[3-5] are time consuming and relatively insensitive, and often complete separation of immunoreactive components is not achieved. The use of an "insulin-specific protease" has been largely discontinued, as it has been shown that degradation of insulin is incomplete and proinsulin may also be degraded.[15] The two-stage assay for proinsulin of Heding[2] is technically more difficult to perform and requires a complete separation of free C-peptide and immobilized proinsulin. The C-peptide antibody has a greater affinity for C-peptide than for proinsulin (Figure 3), so that a small contamination with C-peptide would be measured as a much larger concentration of proinsulin in a radioimmunoassay with proinsulin standards. The appreciable nonspecific binding of peptides to immobilized anti-

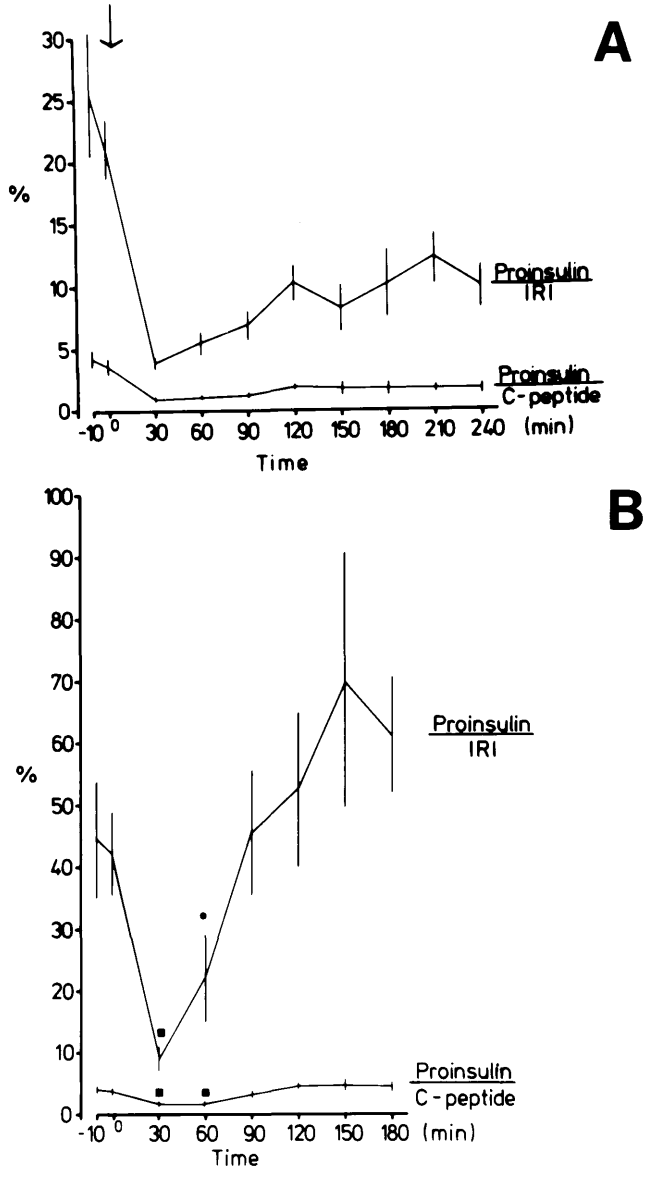


**FIGURE 6. Changes in the immunoreactive proinsulin/IRI ratio and the immunoreactive proinsulin/C-peptide ratio in healthy subjects after oral carbohydrate equivalent to (A) 100 g and (B) 25 g. Significant changes from basal ratios are shown as ■ $P < 0.01$ and ● $P < 0.05$. After 100 g carbohydrate, proinsulin/IRI and proinsulin/C-peptide ratios were suppressed from 30 to 240 min ($P < 0.01$).**

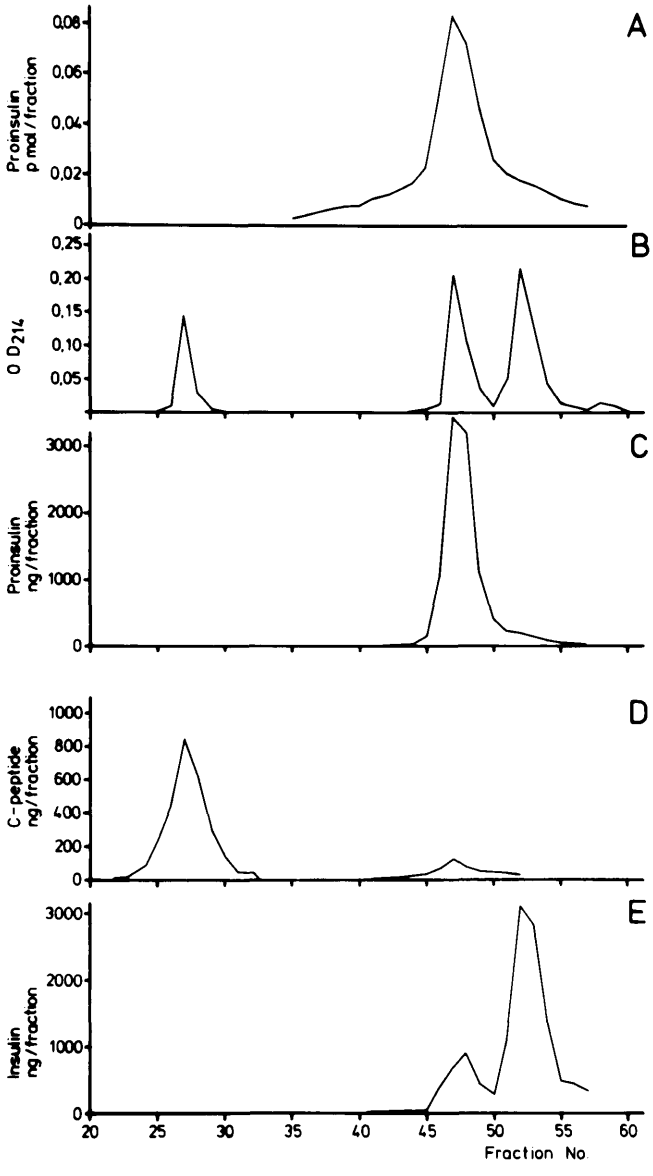


**FIGURE 7. High-performance, ion-exchange chromatography on a Mono Q column of (A) a Sep-Pak extract of endogenous serum proinsulin-like immunoreactivity released after 100 g oral carbohydrate and (B–E) a mixture of synthetic proinsulin (retention time 12.0 min), C-peptide (5.3 min), and insulin (13.7 min). The absorbance at 214 nm was measured (B) and fractions were analyzed at appropriate dilution by radioimmunoassay using assays for proinsulin (A and C), C-peptide (D), and insulin (E).**

bodies has been previously reported.[16] The use of $^{125}$I-proinsulin tracer minimizes assay artefacts that may arise when an antibody has a vastly different affinity for labeled and unlabeled antigen. Similarly, the ethanol precipitation step removes high-molecular-weight plasma components that may nonspecifically inhibit the binding of antigen to antibody[17] and permits concentration of sample when low levels of hormone are to be expected.

Quantitation of serum proinsulin by the two-stage radioimmunoassay method of Heding et al.[7] is complicated by the fact that metabolites of proinsulin also circulate.[18,19] These components may represent intermediates in the conversion of proinsulin to insulin, differing in the site of attachment of C-peptide,[18,19] or may represent the product of a chymotryptic cleavage in the mid-portion of C-peptide.[20] The components display different affinities for antibody in assays for insulin and C-peptide.[21] After chromatography on a Mono Q (anion-exchange) column, the proinsulin antibody Go 4 detected only one component in extracts of human serum with the retention time of proinsulin. The result may indicate that the antibody is specific for intact proinsulin, but further experiments, using structurally defined metabolites, are required to verify this hypothesis. A recent study by Gray et al.[21] has shown that mild trypsinization of biosynthetic human proinsulin increased the proinsulin-like immunoreactivity 20-fold when measured in an immunometric assay using C-peptide antiserum. It was suggested, therefore, that this assay measures primarily the split forms of proinsulin in serum or pancreatic extracts rather than the intact proinsulin molecule. In contrast, in the present study, trypsinization of proinsulin under the same conditions as in ref. 21 resulted in a marked decrease in proinsulin-like immunoreactivity measured in an assay using antiserum Go 4, suggesting that metabolites of proinsulin do not react preferentially with the antiserum.

The levels of serum proinsulin in healthy, fasted subjects reported in this study are in good agreement with those in more recent studies, e.g., 0.009 ± 0.005 pmol/ml,[7] 0.014 ± 0.009 pmol/ml,[22] and 0.023 ± 0.012 pmol/ml.[23] Similarly, the proinsulin/IRI molar ratio in the fasted state in this study (31 ± 4%) agrees well with the value of 20 ± 10% determined by the method of Heding[7] and the value of approximately 20% determined by gel filtration.[4] In view of the differing experimental protocols adopted, it is difficult to compare exactly the proinsulin responses to oral carbohydrate in this study with previous reports. The response to 25 g glucose observed in the present study (31% increase over basal) is much less than the >300% rise reported by Madsbad et al.[14] in response to the same load. As previously discussed, the high levels of circulating C-peptide and split forms of proinsulin after oral glucose may interfere in the two-stage assay of Heding, but not using a proinsulin-specific antibody. Similarly, a recent report[24] comparing human proinsulin standards has shown that radioimmunoassays using a widely available standard prepared from human pancreas at the University of Chicago overestimate proinsulin concentrations by a factor of three compared with a biosynthetic human proinsulin standard.

In agreement with previous reports,[4,7] the proinsulin/IRI and the proinsulin/C-peptide ratios fell rapidly after both high and low oral carbohydrate, followed by a return to basal values in the case of the low load. In view of the large difference in the apparent half-life of insulin ($t_{1/2}$ = 4.8 min) and proinsulin ($t_{1/2}$ = 17.2 min), the molar ratio in peripheral plasma does not reflect directly the rates of secretion.[25] The half-life of C-peptide (20.1 ± 4.0 min) is, however, comparable to that of proinsulin so that the fall in the proinsulin/C-peptide ratio indicates a preferential release of insulin.[26] Small changes in basal insulin concentrations produce large variations in the proinsulin/IRI ratio (Figure 6) and so the proinsulin/C-peptide ratio provides a more reproducible index of secretion. Thus, consistent with previous studies using rat islets,[27,28] the initial preferential release of insulin probably represents exocytosis from mature secretory granules. Approximately two-thirds of the total insulin in rat islets is, however,

stored in an older, more stable compartment and is less readily mobilized for secretion. The delayed increase in serum proinsulin concentrations may represent release from this storage pool. It has been demonstrated by Track et al.[27] that newly synthesized proinsulin is transported through the cell in a vesicular system to the periphery for preferential release, but that this newly synthesized material constitutes only 1% of the total released in a 120-min period.

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**REFERENCES**

[1] Frank, B. H., Pettee, J. M., Zimmerman, R. E., and Burck, P. J.: The production of human proinsulin and its transformation to human insulin and C-peptide. *In* Peptides, Structure and Biological Function. Rockford, Pierce Chemical Co., 1981:729–38.

[2] Podlecki, D. A., Frank, B. H., and Olefsky, J. M.: In vitro characterization of biosynthetic human proinsulin. Diabetes 1984; 33:111–18.

[3] Melani, F., Rubenstein, A. H., and Steiner, D. F.: Human serum proinsulin. J. Clin. Invest. 1970; 49:497–507.

[4] Gorden, P., Sherman, B., and Roth, J.: Proinsulin-like component of circulating insulin in the basal state and in patients and hamsters with islet cell tumors. J. Clin. Invest. 1971; 50:2113–22.

[5] Horwitz, D. L., Starr, J. I., Mako, M. E., Blackard, W. G., and Rubenstein, A. H.: Proinsulin, insulin and C-peptide concentrations in human portal and peripheral blood. J. Clin. Invest. 1975; 55:1278–83.

[6] Kitabchi, A. E., Duckworth, W. C., Brush, J. S., and Heinemann, M.: Direct measurement of proinsulin in human plasma by the use of an insulin-degrading enzyme. J. Clin. Invest. 1971; 50:1792–99.

[7] Heding, L. G.: Specific and direct radioimmunoassay for human proinsulin in serum. Diabetologia 1977; 13:467–74.

[8] Heding, L. G.: Radioimmunological determination of human C-peptide in serum. Diabetologia 1975:541–48.

[9] Fraker, P. J., and Speck, J. C.: Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochem. Biophys. Res. Commun. 1978; 80:849–57.

[10] Melani, E., Ditschuneit, H., Bartelt, K. M., Friedrich, H., and Pfeiffer, E. F.: Über die radio-immunologische Bestimmung von Insulin im Blut. Klin. Wochenschr. 1965; 43:1000–1007.

[11] O'Connor, F. A., Conlon, J. M., Buchanan, K. D., and Murphy, R. F.: The use of perfused rat intestine to characterize the glucagon-like immunoreactivity released into serosal secretions following stimulation by glucose. Horm. Metab. Res. 1979; 11:19–23.

[12] Eng, J., and Yalow, R. S.: Evidence against extrapancreatic insulin synthesis. Proc. Natl. Acad. Sci. USA 1981; 78:4576–78.

[13] Stadil, F., and Rehfeld, J. F.: Preparation of $^{125}$I-labelled synthetic human gastrin I for radioimmunoanalysis. Scand. J. Clin. Lab. Invest. 1972; 30:361–68.

[14] Madsbad, S., Kehlet, H., Hilsted, J., and Tronier, B.: Discrepancy between plasma C-peptide and insulin response to oral and intravenous glucose. Diabetes 1983; 32:436–38.

[15] Starr, J. I., Juhn, D. D., Rubenstein, A. H., and Kitabchi, A. E.: Degradation of insulin in serum by insulin-specific protease. J. Lab. Clin. Med. 1975; 86:631–37.

[16] Murphy, R. F., Conlon, J. M., Iman, A., and Kelly, G. J. C.: Comparison of non-biospecific effects in immunoaffinity chromatography using cyanogen bromide and bifunctional oxirane as immobilizing agents. J. Chromatogr. 1977; 8:136–40.

[17] Conlon, J. M., Bridgeman, M., and Alberti, K. G. M. M.: The nature of big plasma somatostatin: implications for the measurement of somatostatin-like immunoreactivity in human plasma. Anal. Biochem. 1982; 125:243–52.

[18] Gutman, R. A., Lazarus, N. R., and Recant, L.: Electrophoretic characterization of circulating proinsulin and insulin. Diabetologia 1972; 8:136–40.

[19] de Häen, C., Little, S. A., May, J. M., and Williams, R. H.: Characterization of proinsulin-insulin intermediates in human plasma. J. Clin. Invest. 1978; 62:727–37.

[20] Tager, H. S., Emdin, S. O., Clark, J. L., and Steiner, D. F.: Studies on the conversion of proinsulin to insulin. II. Evidence for a chymotrypsin-like cleavage in the connecting peptide region of insulin precursors in the rat. J. Biol. Chem. 1973; 248:3476–82.

[21] Gray, I. P., Siddle, K., Docherty, K., Frank, B. H., and Hales, C. N.: Proinsulin in human serum: problems in measurement and interpretation. Clin. Endocrinol. 1984; 21:43–47.

[22] Heding, L. G.: Insulin, C-peptide and proinsulin in nondiabetics and insulin-treated diabetics. Diabetes 1978; 27 (Suppl. 1):178–83.

[23] Sestoft, L., and Heding, L. G.: Hypersecretion of proinsulin in thyrotoxicosis. Diabetologia 1981; 21:103–104.

[24] Kruse, V., Heding, L. G., Jorgensen, K. H., Tronier, B., Christensen, M., Thim, L., Frank, B. H., Root, M. A., Cohen, R. M., and Rubenstein, A. H.: Human proinsulin standards. Diabetologia 1984; 27:414–15.

[25] Starr, J. I., and Rubenstein, A. H.: Metabolism of endogenous proinsulin and insulin in man. J. Clin. Endocrinol. Metab. 1974; 38:305–308.

[26] Kuzuya, T., and Matsuda, A.: Disappearance rate of endogenous human C-peptide from blood. Diabetologia 1976; 12:519–21.

[27] Track, N. S., Frerichs, H., and Creutzfeldt, W.: Release of newly synthesised proinsulin and insulin from granulated and degranulated isolated rat pancreatic islets. The effect of high glucose concentration. Horm. Metab. Res. 1974; 5:97–103.

[28] Gold, G., Gishizky, M. L., Chick, W. L., and Grodsky, G. M.: Contrasting patterns of insulin biosynthesis, compartmental storage and secretion: rat tumor versus islet cells. Diabetes 1984; 33:556–61.