

Sensitive and specific two-site immunoradiometric assays for human insulin, proinsulin, 65–66 split and 32–33 split proinsulins

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Monoclonal antibody-based two-site immunoradiometric assays are described for human insulin, proinsulin, 65–66 split and 32–33 split proinsulin. The detection limits of the assays lie in the range 0.8–2.5 pM. The assays for 65–66 and 32–33 split proinsulins do not distinguish between these substances and their respective C-terminal di-desamino derivatives. The assay of 65–66 split proinsulin does not cross-react with insulin, proinsulin or 32–33 split proinsulin. This material was undetectable (< 1.0 pM) in plasma taken after an overnight fast in eight normal male subjects and the maximum individual concentration reached in plasma taken during an oral glucose tolerance test of these subjects was 3.8 pM. The proinsulin assay cross-reacted 66% with 65–66 split proinsulin but not with insulin or 32–33 split proinsulin. The 32–33 split proinsulin assay cross-reacted 84 and 60% with proinsulin and 65–66 split proinsulin respectively. The insulin assay cross-reacted 5.3, 62 and 5.0% with intact proinsulin, 65–66 split proinsulin and 32–33 split proinsulin respectively. The very low concentration of 65–66 split proinsulin meant that this derivative did not interfere significantly with the specificity of the assays of proinsulin and insulin. The concentration of 32–33 split proinsulin could be calculated by subtracting the cross-reactivity of the measured proinsulin. The mean concentrations of insulin, proinsulin and 32–33 split proinsulin in eight young male subjects in the fasting state were (pM ± S.E.M.) 20 ± 0.3, 2.3 ± 0.3 and 2.1 ± 0.7 and at the maximum reached during an oral glucose tolerance test, 150 ± 26, 9.9 ± 1.4 and 19.7 ± 6.0 respectively.

INTRODUCTION

The introduction of the radioimmunoassay of insulin (Yalow & Berson, 1959) provided a sensitive means of measuring insulin in human plasma in large numbers of samples. However, the subsequent discovery of proinsulin (Steiner & Oyer, 1967), the demonstration that proinsulin-like molecules exist in human plasma (Roth *et al.*, 1968; Given *et al.*, 1985) and that human proinsulin reacts like human insulin in many human insulin radioimmunoassay methods (Heding, 1972) has put in question the specificity of human plasma insulin radioimmunoassays. This problem of specificity is unlikely to be serious in the assay of insulin in plasma from normal subjects in which it has been estimated that proinsulin-like molecules account for only 10–20% of the immunologically insulin-like molecules in plasma. However, in plasma from diabetic subjects, proinsulin-like molecules are present in higher concentration (Mako *et al.*, 1977). Since their biological, insulin-like activity is very much lower than that of insulin (Peavy *et al.*, 1985) their measurement as 'insulin' may lead to incorrect conclusions concerning the availability of biologically active insulin in diabetes.

Several years ago we introduced the idea of using labelled antibodies as an alternative procedure to radioimmunoassay for the provision of highly sensitive assays

(Miles & Hales, 1968). We then developed this approach into an assay format employing two antibodies which we termed a two-site immunoradiometric assay (Woodhead *et al.*, 1974). We suggested that the use of the binding specificities of two antibodies should greatly enhance assay specificity. At that time a major disadvantage of procedures using labelled antibodies was the need for a relatively large amount of pure antibody. In an attempt to reduce this problem, we devised the indirect two-site immunoradiometric assay (Beck & Hales, 1975) and used this approach to establish an assay for human proinsulin (Rainbow *et al.*, 1979). However, we subsequently discovered that this assay detected partially processed proinsulins and did not recognize intact proinsulin (Gray *et al.*, 1984). We were, therefore, confronted with another problem of assay specificity, namely the need to distinguish specifically the individual insulin-like molecules in plasma—insulin, intact proinsulin, and partially processed proinsulin. The processing of human proinsulin is believed to occur by endoproteolysis at the junctions of amino acids 32 and 33 and 65 and 66 to yield respectively 32–33 and 65–66 split proinsulin. Removal of the pairs of basic amino acids thereby exposed at the new C-terminal positions is thought to be carried out by a carboxypeptidase and produces respectively des-31–32- and des-64–65-proinsulin.

The ability to approach this problem has been revo-

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lutionized by the introduction of the monoclonal antibody technique (Kohler & Milstein, 1975) and by the bioengineered production of human proinsulin (Frank *et al.*, 1981). We have, therefore, attempted to produce a panel of monoclonal antibodies with different binding specificities for these immunologically insulin-like molecules, such that their use in different combinations would allow us to determine specifically the individual concentrations of these molecules. We have reported our early attempts to assay intact proinsulin by this means (Gray *et al.*, 1987). We now report the successful outcome of the overall objective, together with the concentrations of insulin, intact, and partially processed proinsulins in plasma from normal male subjects.

MATERIALS

Reagents

Intact, 65–66 split, des-64–65-, 32–33 split and des-31–32 human proinsulins were synthesized, purified by h.p.l.c. and, where appropriate, iodinated, by Lilly Research Laboratories, Indianapolis, IN 46285, U.S.A. (Frank *et al.*, 1981). Human insulin standard (Actrapid) was a gift from Novo Industri A/S and anti-human C-peptide antibody PEP 001 was a gift from Novo Laboratories, Bagsvaerd, Denmark. All chemicals were obtained from Sigma Chemical Co., and tissue culture medium and reagents were purchased from Gibco Europe.

METHODS AND RESULTS

Preparation and selection of monoclonal antibodies

Mouse monoclonal antibodies reacting with human insulin and intact proinsulin were obtained by fusing mouse spleen cells with NSO mouse myeloma cells, which were a gift from Dr. C. Milstein (Galfre & Milstein, 1981). The mouse spleen cells used to produce monoclonal antibody A6 were obtained from a mouse immunized by subcutaneous injection with intact human proinsulin (20 µg) emulsified with Freund's complete adjuvant. A boost immunization (20 µg) was administered intravenously after 6 weeks and again after a further 10 weeks. The final boost immunization (20 µg) was administered by intravenous injection 1 year later and 4 days before fusion. This immunization protocol was one of a series utilized in these experiments with various intervals between primary and booster injections. However, the number of fusions carried out with spleens from animals following each of the protocols tried was not sufficient for us to conclude that such a protocol was significantly superior to more rapid procedures. To obtain the monoclonal antibody 14B, a mouse was immunized by subcutaneous injection with human insulin (100 µg) emulsified with Freund's complete adjuvant. After 6 weeks, another subcutaneous immunization with insulin (100 µg) emulsified with Freund's incomplete adjuvant was administered. After a further 18 weeks, a boost immunization with insulin (20 µg) was administered by intraperitoneal injection 4 days before fusion. The mouse serum and cell supernatants were assayed as below. The monoclonal antibody 3B1 was prepared as previously described (Gray *et al.*, 1987).

Serum obtained from mice before fusion was performed, and supernatants from the cells after fusing, were assayed by incubating the serum or supernatant (100 µl) with ¹²⁵I-labelled human proinsulin (210 µCi/µg)

or ¹²⁵I-human insulin (150–200 µCi/µg, 100 µl, approx. 20000 c.p.m.) for 24 h at 4 °C, or 3 h at room temperature, and then adding cellulose-bound sheep anti-(mouse IgG) antibody (50 µl of a 5 mg/ml cellulose suspension) and incubating for 30 min at room temperature (Soos & Siddle, 1982). Ice-cold 0.025 M-sodium barbitone buffer, pH 8 (1 ml) (0.05 M-sodium barbitone buffer is 0.05 M-sodium barbitone/0.09 M-NaCl/0.0015 M-NaN₃/0.014 M-HCl/0.5% bovine serum albumin, pH 8.0; diluted with distilled water) was added and then centrifuged at 1700 g (Fison Coolspin) for 10 min. The supernatant was discarded, the pellet was washed again with 1 ml of ice-cold 0.025 M-sodium barbitone buffer, pH 8, centrifuged and the supernatant again discarded. The pellet was counted for 1 min using a Nuclear Enterprises 1600 Gamma counter of ¹²⁵I counting efficiency approx. 70%.

The monoclonal antibodies A6 and 14B were selected to bind either intact human proinsulin or human insulin respectively to the exclusion of the other. All hybrid cell lines were routinely cloned at least twice before injection into mice to produce ascitic tumours (Galfre & Milstein, 1981). Antibodies for cellulose immunoadsorbent preparation were partially purified from ascites fluids by precipitation with 40% (v/v) saturated (NH₄)₂SO₄. Antibodies for iodination were further purified on a DEAE-Affigel Blue (BioRad) column and eluted with an NaCl gradient from 0 to 500 mM (Bruck *et al.*, 1982). The antibodies were shown to be pure by electrophoresis on a 10% SDS/polyacrylamide gel.

The affinity of the monoclonal antibodies used in these assays was investigated using Scatchard analysis (Scatchard, 1949) essentially as described by Soos & Siddle (1982). The association constants of the monoclonal antibodies used were: A6, 4 × 10⁹ M⁻¹ for proinsulin; 3B1, 8.0 × 10⁹ M⁻¹ for proinsulin and 1.4 × 10¹⁰ M⁻¹ for insulin; 14B, 1.1 × 10⁸ M⁻¹ for insulin. A6 did not react with ¹²⁵I-labelled human insulin, and 14B did not react with ¹²⁵I-labelled human intact proinsulin. All three monoclonal antibodies were IgG₁.

Preparation of cellulose immunoadsorbents

Partially purified monoclonal antibodies were coupled to finely divided aminocellulose by diazotization (Hales & Woodhead, 1980), reacting 100 mg of protein with 100 mg of cellulose. The resulting immunoadsorbents contained approx. 200 µg of protein per mg of cellulose and had the following binding capacities: cellulose-linked 3B1 bound approx. 4 µg of insulin per mg of cellulose, and cellulose-linked A6 and 14B bound approx. 2.5 µg of proinsulin and insulin respectively per mg of cellulose. The immunoadsorbents were stored in a suspension of 5 mg of cellulose/ml in 0.05 M-sodium barbitone buffer, pH 8.0, and were washed twice with the same buffer immediately before use, in order to remove any unbound antibody, and were then diluted as required for a particular assay.

Iodination of antibodies

Purified monoclonal antibodies were iodinated by the iodogen method (Salacinski *et al.*, 1981) using 20 µg of antibody and 0.5 mCi of Na¹²⁵I (Amersham International) with 10 µg of iodogen (Pierce) in 50 µl of 0.5 M-sodium phosphate, pH 7.5, for 15 min at room temperature. Unreacted iodide was separated on a 1 cm × 25 cm column of Sephadex G-25 (Pharmacia)

eluted with 0.05 M-sodium barbitone buffer, pH 8.0. The specific radioactivity of antibodies was 2.5–3.0 $\mu\text{Ci}/\text{pmol}$ (18–22 $\mu\text{Ci}/\mu\text{g}$).

Collection of human plasma

Samples of blood from eight non-diabetic, non-obese healthy male subjects, age range 22–36 years, were collected into heparinized tubes before and during a 75 g oral glucose tolerance test and centrifuged at 1800 g (Baird and Tatlock Auto Bench Centrifuge mark IV) at room temperature for 5 min. The plasma was stored at -20°C until assayed.

Standards

Standards were made up in 0.05 M-sodium barbitone buffer, pH 8, containing 5% (w/v) bovine serum albumin and stored at -20°C until used. In no system was any difference detected between the behaviour of 65–66 split and des-64–65 human proinsulin, nor between 32–33 split and des-31–32 human proinsulin. However, in view of the high activity of carboxypeptidase H in the insulin secretory granule (Davidson & Hutton, 1987) it was decided to use des-64–65 or des-31–32 human proinsulins routinely as standards for the respective assays of the split proinsulins. Since we do not know to what extent the 65–66 or 32–33 human split proinsulins are converted to des-65, des-64–65, or des-32, des-31–32 human proinsulins respectively, the concentrations quoted in plasma are strictly speaking the sum of all the products of the respective initial endopeptidase cleavage plus further carboxypeptidase removal of the one or two C-terminal basic amino acids revealed by the endopeptidase cleavage.

Assay optimization

The following points were established (results not shown): two successive periods of overnight incubation with either the labelled antibody or the cellulose-bound antibody followed by the other gave optimum sensitivity; there was no significant improvement when two periods of 48 h incubation were used; assay performance was less good if the labelled antibody and the cellulose-bound antibody were added simultaneously; assay sensitivity was optimal when the incubation and washing were carried out at 4°C but the degree of improvement compared with room temperature varied with the assay; 200 μl samples of standard or unknown gave greater sensitivity than 100 or 50 μl , larger volumes were not tested as they would demand excessive amounts of blood to be taken in clinical situations; additional washing steps lowered both the non-specifically and the specifically bound labelled antibody with no improvement in assay performance.

Recovery and dilution

Recovery from plasma was assessed by the addition of known quantities of the appropriate hormones to plasma samples. Recovery of human insulin (50–100 pM) was 78% (range 69–91%) at 1:4 dilution of normal plasma samples. Under the same conditions recoveries of intact, 65–66 and 32–33 split proinsulins (5–100 pM) were 77% (60–91%), 80% (68–99%) and 75% (70–80%) respectively. Similarly, plasma samples were serially diluted to ensure that these dilution curves were parallel to the standard curves. It was observed that for the assay of insulin the dilution curves were only parallel to the insulin standard curve at dilutions of 1:4 and greater.

Other dilution curves were parallel to the standard curve throughout the range of dilution.

Inter- and intra-assay variation

The intra-assay precision of the assays as determined by replicate analysis of samples gave coefficients of variation of $< 10\%$ over the concentration ranges 20–476 pM, 22–930 pM, 12–152 pM and 8–230 pM for insulin, intact proinsulin, 65–66 and 32–33 split proinsulin.

The inter-assay precision (coefficient of variation) determined from the mean of duplicate assays in different assays was $< 15\%$ over the concentration ranges 23–127 pM and 2.2–9.4 pM for insulin and intact proinsulin. Precision was also assessed by the method of Ekins (1983) from the precision of duplicates in at least 10 assays. Coefficients of variation were $< 15\%$ over the concentration ranges 3–240 pM, 0.5–50 pM, 2.5–15 pM and 12–30 pM for insulin, intact proinsulin, 65–66 and 32–33 split proinsulin.

Two-site assay of intact proinsulin

Iodinated monoclonal antibody A6 [50 μl , approx. 20000 c.p.m., containing 12% (v/v) of normal mouse serum] was added to either the unknown plasma sample, intact proinsulin standard, or quality control plasma (200 μl), mixed and incubated for 24 h at 4°C . Cellulose-bound monoclonal antibody 3B1 (50 μl , binding approx. 30 ng of insulin/50 μl) was then added, mixed and incubated for a further 24 h at 4°C . At the end of this period, 0.5 ml of ice-cold 0.025 M-sodium barbitone buffer, pH 8, was added to each tube and mixed. The tubes were centrifuged at 8300 g (Beckman Microfuge 12) for 3 min at room temperature and the supernatants removed by aspiration. The pellets were washed again by the addition of 1 ml of ice-cold 0.025 M-sodium barbitone buffer, pH 8, containing Tween 20 (0.5%, v/v) and centrifuging at 8300 g for 3 min. The supernatants were removed by aspiration. The pellets were counted for 10 min in a Nuclear Enterprises 1600 Gamma Counter. Standard curves and unknown values were calculated by using the Nuclear Enterprises RIA Software and an Apple IIe computer.

Since plasma insulin concentrations are normally greatly in excess of those in proinsulin the effect of insulin on the standard curve for proinsulin was tested. Insulin at concentrations up to 3.5 nM had no effect. This was not the case if the assay was carried out in the reverse configuration (using labelled 3B1 and cellulose-bound A6) when the addition of insulin reduced the slope of the standard curve.

Fig. 1 shows a typical standard curve for the measurement of intact human proinsulin together with the cross reaction with 65–66 split human proinsulin ($66 \pm 3\%$). Human insulin (see above) or 32–33 split human proinsulin at concentrations up to 50 pM (which was the highest tested) were undetectable in the assay. The assay detected 1.8 pM intact proinsulin (mean \pm 3 S.D. of the zero signal).

Two-site assay of human insulin

The assay for human insulin was carried out as described above using iodinated monoclonal antibody 3B1 (50 μl , approx. 20000 c.p.m., containing 12% (v/v) normal mouse serum) and cellulose-bound monoclonal antibody 14B (50 μl , binding approx. 30 ng of insulin/

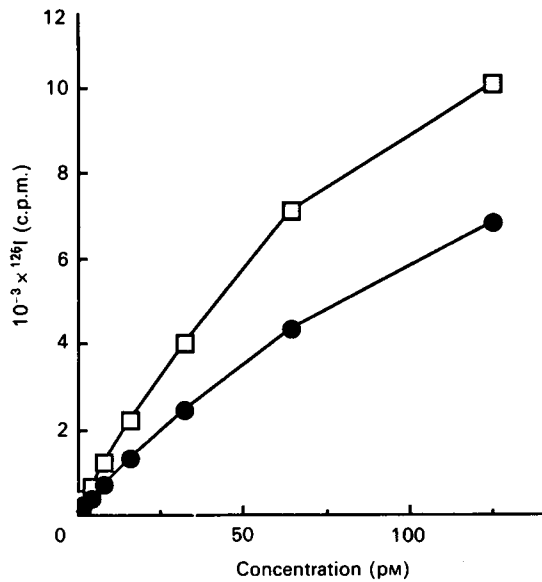


Fig. 1. Assay of human intact proinsulin

Standard curve of human intact proinsulin (□) assayed with iodinated antibody A6 and cellulose-bound antibody 3B1 as described in the text. The cross-reactivity of 65–66 split proinsulin (●) is also shown. Human insulin and 32–33 split proinsulin did not react in this assay.

50 μ l). The pellets were washed as for the proinsulin assay with the addition of a further wash of 1 ml of ice-cold 0.025 M-sodium barbitone buffer, pH 8, containing Tween 20 (0.5%, v/v), then centrifuging and aspirating as previously described. The pellets were counted as above.

A typical standard curve is shown in Fig. 2. The cross-reactivities with intact proinsulin, 65–66 split proinsulin and 32–33 split proinsulin were $5.3 \pm 1.4\%$, $62 \pm 6\%$, and $5 \pm 1\%$ respectively. At high insulin concentrations (> 50 pM) high concentrations of proinsulin (> 25 pM) caused a suppression of the measured signal. Dilution of samples 1:4 overcame this effect. The assay detected 2.3 pM human insulin (mean + 3 S.D. of the zero signal).

Two-site assay for 65–66 split proinsulin

The competitive binding studies of monoclonal antibodies A6 and 14B indicated they could be used in a specific assay for 65–66 split proinsulin similar to that for intact proinsulin by replacing the cellulose-bound 3B1 with cellulose-bound 14B. Further investigation showed that, although 14B did not bind to intact proinsulin in a competitive binding assay, in the presence of A6, 14B bound intact proinsulin. (The association constant of 14B for proinsulin in the presence of A6 was $4 \times 10^8 \text{ M}^{-1}$ and in the absence of A6 it was not measurable.) It was, therefore, necessary to remove intact proinsulin before the addition of A6. Thus the cellulose-bound monoclonal antibody 14B (50 μ l, binding approx. 30 ng of insulin/50 μ l) was added to the unknown plasma sample, standard or quality control plasma (200 μ l), mixed and incubated for 24 h at 4 °C. Then 0.5 ml of ice-cold 0.025 M-sodium barbitone buffer, pH 8, was added to each tube and mixed, the tubes were centrifuged at 8300 g for 3 min at room temperature and the supernatants were removed by aspiration. Iodinated monoclonal antibody

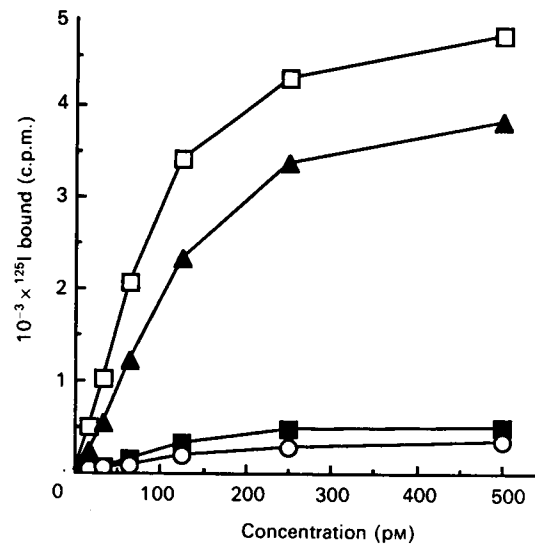


Fig. 2. Assay of human insulin

Standard curve of human insulin (□) assayed with iodinated antibody 3B1 and cellulose-bound antibody 14B as described in the text. The cross-reactivities of human intact proinsulin (■), 32–33 split proinsulin (○) and 65–66 split proinsulin (▲) are also shown.

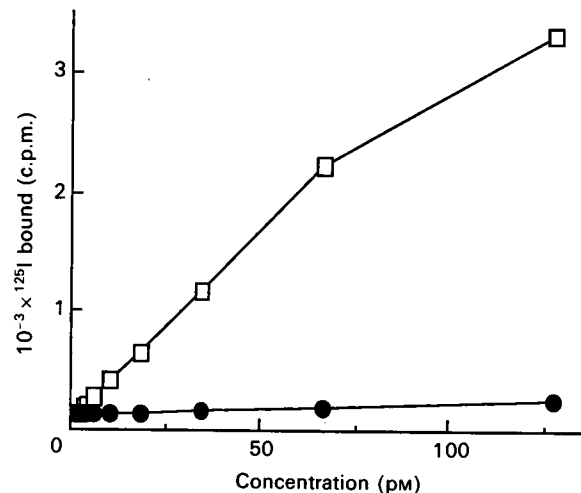


Fig. 3. Assay of human 65–66 split proinsulin

Standard curve of human 65–66 split proinsulin (□) assayed with cellulose-bound antibody 14B and iodinated antibody A6 as described in the text. The cross-reactivity of intact proinsulin (●) is also shown. Human insulin and 32–33 split proinsulin did not react in this assay.

A6 [50 μ l, approx. 20000 c.p.m., containing 12% (v/v) of normal mouse serum] was then added to each tube, mixed and incubated for 24 h at 4 °C. Then 0.5 ml of ice-cold 0.025 M-sodium barbitone buffer was added to each tube, mixed, centrifuged and the supernatants removed by aspiration. The pellets were washed twice more as for the insulin assay.

A typical standard curve is shown in Fig. 3. Human insulin at concentrations up to 1 nM and 32–33 split proinsulin at concentrations up to 128 pM, the highest tested, were undetectable in this assay. The cross-

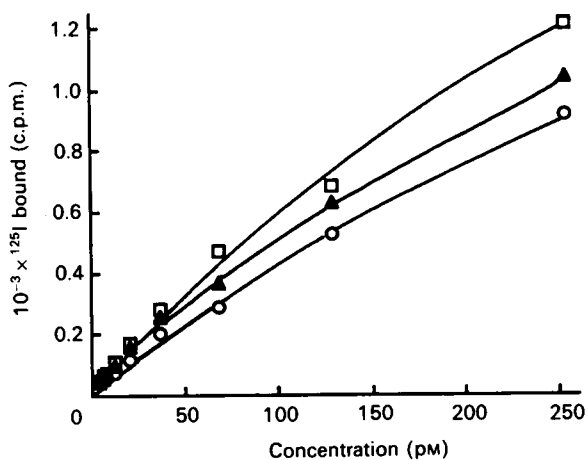


Fig. 4. Assay of human 32-33 split proinsulin

Standard curve of human 32-33 split proinsulin (□) assayed with cellulose-bound antibody 3B1 and iodinated anti-(C-peptide). The cross-reactivities of 65-66 split proinsulin (○) and intact proinsulin (▲) are also shown. Human insulin did not react in this assay.

activity with intact proinsulin was $6 \pm 1\%$. The assay detected 0.8 pM 65-66 split human proinsulin (mean + 3 S.D. of the zero signal).

Two-site assay for 32-33 split proinsulin

The assay was carried out essentially as described for the 65-66 split assay using cellulose-bound antibody 3B1 and iodinated anti-(C peptide) antibody PEP 001, but the cellulose-bound antibody pellets were washed three times before adding the iodinated antibody to remove the potentially large excess of C-peptide. A typical standard curve is shown in Fig. 4. Human insulin at concentrations up to 500 pM , which was the highest tested, did not react in this assay. The cross-reactivities of intact proinsulin and 65-66 split proinsulin were $84 \pm 2\%$ and $60 \pm 5\%$ respectively. The assay detected 2.5 pM 32-33 split proinsulin (mean + 3 S.D. of the zero signal).

Concentrations of 65-66 split proinsulin, insulin, intact and 32-33 split proinsulin in human plasma

The results of assays carried out on eight normal male subjects during an oral glucose tolerance test are shown

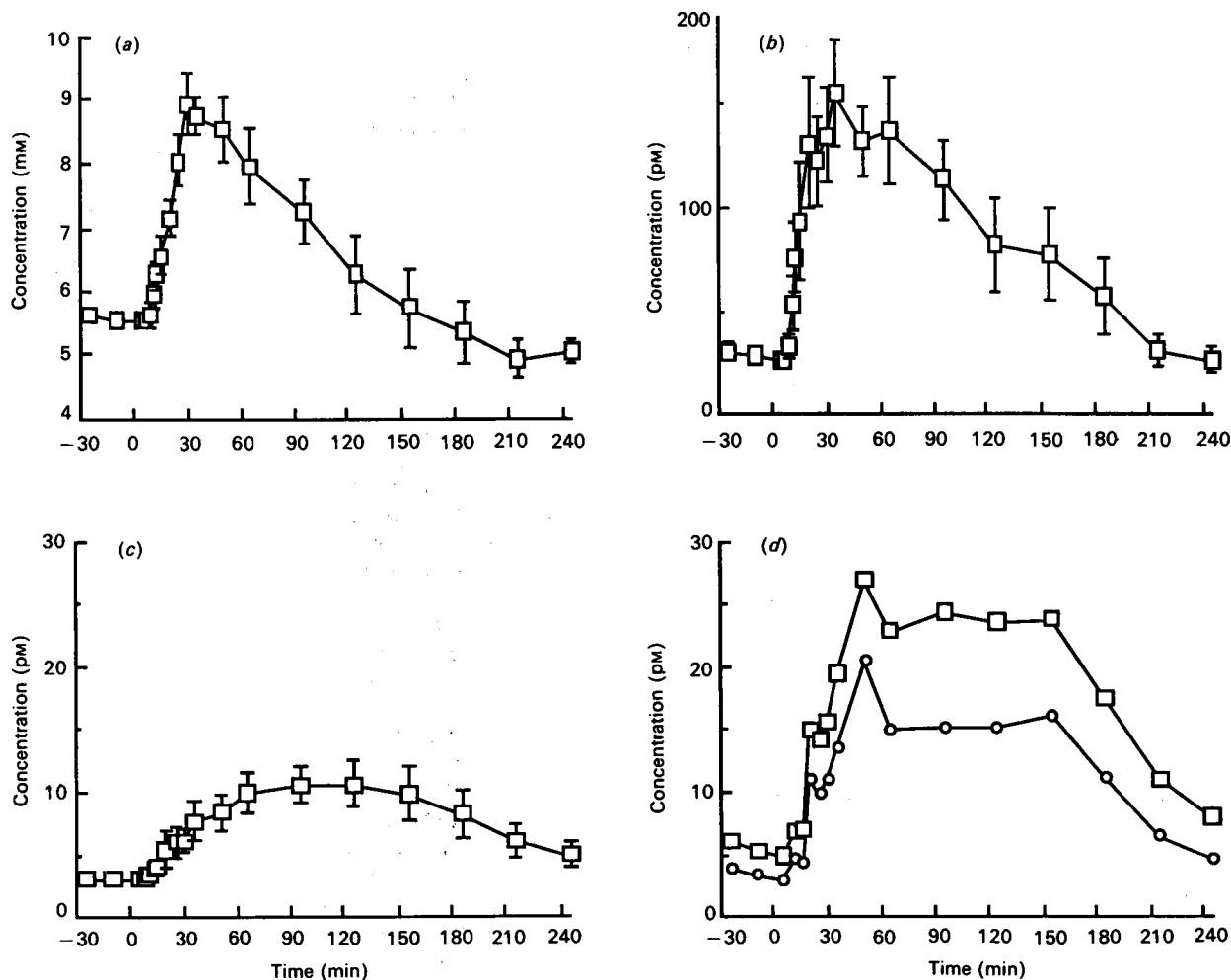


Fig. 5. Assay of insulin-related molecules during normal oral glucose tolerance tests

Results of assays on the blood and plasma of eight normal weight, non-diabetic, young males during an oral (75 g) glucose tolerance test. Concentrations (mean \pm S.E.M.) of (a) blood glucose, (b) plasma insulin, (c) plasma intact proinsulin, and (d) plasma 32-33 split proinsulin are shown. □, Total hormone assayed; ○, values corrected for cross-reaction of intact proinsulin. S.E.M. values are omitted for clarity.

in Fig. 5. The fasting concentration of 65–66 split proinsulin was undetectable (< 1 pM) in all eight of the subjects and remained undetectable post-glucose in two of the subjects. The maximum concentration observed after oral glucose was 3.8 pM at 60 min with a mean maximum concentration of 2.2 ± 0.2 pM for the six subjects with detectable levels. Therefore, because the concentrations were so low, it was not necessary to remove the contribution of this intermediate from the assays for insulin or intact proinsulin either by subtraction or by adsorption prior to assay.

The concentration of insulin from a basal concentration of 20 ± 3.6 pM closely followed that of glucose, having doubled by 6 min, peaked at 30 min (7.5-fold above the fasting concentration) and returned to the fasting concentration at 210 min. Intact proinsulin, on the other hand, rose more slowly from a basal concentration of 2.3 ± 0.3 pM, doubled by 15 min, reached a plateau between 60 and 150 min (4-fold above the fasting concentration) and was still approximately double the fasting concentration 240 min after glucose. The concentration of 32–33 split proinsulin, corrected for the cross-reaction of intact proinsulin in the assay, showed changes intermediate between those of insulin and intact proinsulin. The concentration from a basal concentration of 2.1 ± 0.7 pM quadrupled by 10–15 min, rising to a peak at 45 min (9-fold above the fasting) and returning close to the fasting concentration at 240 min.

The percentage of the total insulin-like molecules accounted for by intact or partially processed proinsulin was 18% in the fasting state and 25%, 27% and 27% at 120, 180 and 240 min after glucose respectively.

DISCUSSION

Many attempts to improve the sensitivity and specificity of insulin assays have been made. However, we are not aware of any assays of insulin which have been characterized in detail with regard to their cross-reactivity with the proinsulin-like molecules and which in particular have been shown to give operational specificity for insulin. When cross-reactivity with proinsulin-like molecules has been assessed it has been studied using intact proinsulin (Yoshioka *et al.*, 1979; Ruan *et al.*, 1986; Tolvonen *et al.*, 1986; Comitti *et al.*, 1987).

Similarly, several radioimmunoassays for proinsulin have been reported and some detailed studies of their specificities have been carried out. All assays reported thus far either lack sufficient sensitivity to allow the measurement of the individual proinsulin-like molecules in plasma from normal fasting subjects without extraction and concentration (Cohen *et al.*, 1985) or do not have the specificity to discriminate between the individual proinsulin-like molecules present in the circulation (Deacon & Conlon, 1985; Hartling *et al.*, 1986; Ward *et al.*, 1986). We have previously shown (Gray *et al.*, 1984) that much of the material detected in plasma as 'proinsulin' is partially processed proinsulin. We now show that nearly all the partially processed proinsulin is 32–33 split proinsulin and that the concentration of this material may be as high as, or higher than, that of intact proinsulin in the plasma of normal subjects. In recent studies of subjects with Type II diabetes mellitus we have found concentrations of both intact and 32–33 split proinsulin during glucose tolerance tests sufficiently high to lead to

spurious conclusions as to their insulin status (Temple *et al.*, 1989).

In the process of establishing the present assays certain interesting technical problems have been encountered. In the first place the sensitivity of the assays for the proinsulin-like molecules is either just adequate (intact proinsulin and 32–33 split proinsulin) or inadequate (65–66 split proinsulin) for the accurate determination of these substances individually in plasma from fasting normal subjects. Further improvement of the sensitivity of these assays is therefore desirable, particularly for studies of the inhibition of the release of these molecules in fasting individuals. It is unlikely that much greater sensitivity could be achieved by the use of directly radioiodinated antibodies. Secondly, the very wide range of concentrations of the insulin-related peptides in plasma (from several hundred to less than one picomolar C-peptide and 65–66 split proinsulin respectively in the normal fasting state) places considerable constraints on the configuration of the assays. It is often necessary to use a solid-phase antibody preparation with a very high binding capacity to avoid its saturation by potentially cross-reacting materials. In other situations it is necessary to use several washes to eliminate the problem of excess cross-reacting material and this inevitably reduces assay precision (e.g. the removal of free C-peptide from the 32–33 split proinsulin assay). Thirdly, it is not always possible to predict the specificity of monoclonal antibody based two-site immunoradiometric assays from the individual specificities of the separate monoclonal antibodies as determined in a competitive-binding radioimmunoassay format. For example, in the latter type of binding study the antibody 14B showed no cross-reaction with 65–66 split proinsulin, yet in the presence of the antibody A6 it bound intact proinsulin with quite a high avidity. It is not clear whether this change of avidity is due to change in the conformation of the antigen induced by antibody binding or to the production of highly stable cyclic complexes (Moyle *et al.*, 1983). Studies with monovalent antibody fragments might discriminate between these possibilities. Finally, even the present assay methods do not discriminate between the split proinsulins and these molecules after they have been subjected to the action of carboxypeptidase.

The results of the assays carried out in a group of normal relatively young male subjects provide a considerable amount of new information. The mean fasting insulin concentration 20 pM (2.9 munit/l) is somewhat lower than that reported in any previous study. We believe that this is due to the age of the subjects and the improved performance of the assay itself. In a group of older subjects (mean age 53 years) the mean fasting plasma insulin concentration was 28 pM (4.0 munit/l) (our unpublished work). This concentration is in good agreement with that found with the more sensitive of the radioimmunoassays (e.g. O'Rahilly *et al.*, 1988). Some reduction of the measured insulin concentration is due to the removal of cross-reactivity with proinsulin. In the present assay the detection limit for insulin is an order of magnitude lower than the normal fasting insulin concentration, whereas in most radioimmunoassays the limit of detection is close to the normal fasting insulin concentration. Thus the inevitable 'noise' seen at the limit of detection of insulin is less significant using the present assay method.

The total concentration of proinsulin-like molecules

which we have measured in fasting plasma agrees well with that previously determined by less specific assays (Heding, 1977; Rainbow *et al.*, 1979; Cohen *et al.*, 1985; Deacon & Conlon, 1985; Hartling *et al.*, 1986; Ward *et al.*, 1986). The finding that 32–33 split proinsulin is the major proinsulin-like molecule present in plasma during a glucose tolerance test agrees with our previous observations using an assay which did not detect intact proinsulin (Gray *et al.*, 1984). It is of interest that there is 10–15 times more 32–33 split than 65–66 split proinsulin in plasma. This is consistent with the observation that there is a 3-fold excess of the 32–33 split proinsulin in human pancreas (Given *et al.*, 1985) and the higher biological activity and shorter half life of the 65–66 split molecule (Peavy *et al.*, 1985; Tillil *et al.*, 1987). As would be expected from the differences in half-life of insulin, 32–33 split and intact proinsulin, the kinetics of the 32–33 split molecule during a normal oral glucose tolerance test are intermediate between those of insulin and intact proinsulin.

We conclude that the assays reported here represent a significant advance on other methods reported to date. They enable the specific assay of insulin and of the proinsulin-like molecules for the first time. They will, therefore, be of value in determining the true insulin status of individuals, and in the search for abnormalities of insulin synthesis and processing.

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