We describe the application of a stable isotope dilution assay (IDA) to determine precise insulin, C-peptide, and proinsulin levels in blood by extraction from serum and quantitation by mass spectrometry using analogues of each target protein labeled with stable isotopes. Insulin and C-peptide levels were also determined by immunoassay, which gave consistently higher results than by IDA, the relative difference being larger at low concentrations. Insulin, C-peptide, and proinsulin levels were all shown by IDA to be higher in type II diabetics than in non-diabetics, with mean values rising from 22 (±2) to 92 (±8), 335 (±11) to 821 (±24), and 6 (±1) to 37 (±3) pm, respectively. Interestingly, the ratio between IDA and immunoassay values for insulin levels increased from 1.3 in non-diabetics to 1.7 in type II diabetics. The ratio between proinsulin and insulin levels by IDA increased from 0.24 in non-diabetics to 0.36 in type II diabetics, whereas the ratio between C-peptide and insulin levels by IDA decreased from 17.6 to 10.7. This disproportionate change in protein levels between different types of individuals has implications for the metabolism of insulin in the diabetics studied (type II) and suggests that C-peptide levels are not always a reliable guide as to pancreatic insulin secretion. In addition, levels of the 33-residue C-peptide (partially trimmed) were shown to be less than 10% of that of the fully trimmed 31-residue C-peptide levels, and we tested IDA in a clinical context by two post-pancreatic graft studies. IDA was shown to give direct, positive identification of the target protein with unrivalled accuracy, avoiding many of the problems associated with present methodology for protein determination.

At present, protein levels in humans are measured by following radiolabeled material or by the use of immunological reagents. Radioiodination is the most common procedure of protein labeling and has been of great value. However, it has been recognized that chemical modification of a protein in this way can affect its biological and physicochemical behavior. Tissue dehalogenases, which are known to remove iodine from iodotyrosine, may invalidate concentration measurements, particularly of fragments of the target molecule (1, 2). Tritiated proteins have been used since they escape these objections (3), but the ethical issue of injecting radioactive proteins of any kind into human subjects remains under severe scrutiny.

The alternative method involving the use of immunological reagents to estimate blood protein levels remains the best current approach. However, antibodies used in these measurements often cross-react with precursors of the target protein or with its smaller, degraded fragments, creating uncertainty in results (2, 4–6). Although modern forms of radioimmunoassay, microparticle enzyme immunoassay (MEIA),1 and immunoradiometric assay (7–9) reduce many of these cross-reactivities, the methodology still remains an indirect measure of the target molecule, where endogenous and administered exogenous protein cannot be distinguished. Also, pathological samples may contain endogenous antibodies that can interfere with conventional assays and invalidate the results obtained.

Such difficulties, as shown by significant discrepancies between immunoassays performed at different centers on the same samples (10), have led to a feeling that measurements of insulin levels, for example, have been systematically overestimated by radioimmunoassay in type II (non-insulin-dependent) diabetic individuals, resulting in insufficient attention being paid to insulin deficiency as opposed to insulin resistance (5). Indeed, many diseases, including type II diabetes and tumors such as insulinomas or certain other carcinomas, lead to a disproportional increase in the concentration of proteins such as proinsulin and its conversion intermediates that are immunologically cross-reactive with insulin (6, 11–13). The uncertain clinical interpretation of results involving such important diseases remains a serious problem.

A definitive measure of blood protein concentration would be most welcome in calibrating existing procedures and investigating suspect results. To be able to fully understand the fate and distribution of proteins in vivo, a technique is required that can accurately determine each of the components directly involved, allowing a distinction between endogenous and exogenous protein while avoiding the injection of radioactive material. To this aim we describe an alternative technique, referred to as isotope dilution assay (IDA), that allows precise quantification of specific proteins at physiological concentrations in blood.

In this procedure, target proteins are extracted from a 1-ml
Insulin, C-peptide, and Proinsulin Levels in Humans by IDA

Preparation and Purification of Protein Analogues

The proinsulin gene, in a construction containing a polyhistidine tag (27), was cloned and expressed in Escherichia coli by a modified (see “Acknowledgments”) standard procedures (28) at a yield of 5–10 mg/liter of culture. After protein isolation using a 10-ml nickel-Sepharose fast flow column (Pharmacia Biotech Inc.), the polyhistidine tag was removed with cyanogen bromide (1.5 mg/mg of protein) at pH 1, and proinsulin was purified by reverse-phase HPLC (using a Macherey Nagel 300-A C8 column (250 × 10 mm)) with a linear gradient of acetonitrile (22–36% in 30 min) in 0.1% trifluoroacetic acid in water. Proinsulin was labeled biosynthetically with "^15"N by expression of the gene in a culture medium containing "^15"NH4Cl (28, 29), where the incorporation of "^15"N was calculated to be 98.8% by mass spectrometry. Furthermore, proinsulin was double-labeled with both "^2"H and "^15"N by expression in "^15"NH4Cl and 50% "^2"H2O medium, where the final incorporation of "^15"N after protein purification was calculated to be 24.3% (assuming 98.8% "^15"N incorporation). Each proinsulin analogue was cleaved by proteolytic techniques, using trypsin (2.5 μg/mg of protein) and carboxypeptidase B (5 μg/mg of protein) at pH 8 to yield "^14"N- and "^15"N"H-labeled insulin and C-peptide (31- and 33-residue forms). ["^3"H]Phe-"^15"HVal"^14"H insulin was prepared as described previously (30). ["^3"H]Gly, C-peptides ("^15"H-C-peptides) (31- and 33-residue forms) were prepared by total peptide synthesis using standard Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry (Applied Biosystems 430A peptide synthesizer). Subsequent peptide purification was performed by reverse-phase HPLC using similar conditions as described above.

The concentration of proinsulin and insulin solutions was determined from the absorbance at 280 nm (31), and the concentration of C-peptide solutions was determined by amino acid analysis, HPLC, and electrospray ionization mass spectrometry (ESI-MS) against a standard. Protein aliquots were prepared at a concentration of 25 μg/ml, freeze-dried, and stored at −20 °C. To help avoid protein loss, any further protein dilution was carried out in siliconized glass tubes with a solution of bovine serum albumin (1 mg/ml). Our investigations on the C-peptide refer solely to the 31-residue form, unless the 33-residue form is specifically mentioned.

EXPERIMENTAL PROCEDURES

Materials

Carboxypeptidase B (from porcine pancreas) was obtained from Boehringer Mannheim. Trypsin (TPCK-treated, from bovine pancreas), antisera against human insulin (developed in guinea pig), and bovine serum albumin (essentially fatty acid and globulin free) were obtained from Sigma. All other reagents (except the antisera against human C-peptide, see “Acknowledgments”) were of analytical grade and were purchased from either Fluka Chemie AG or Sigma.

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The proinsulin gene, in a construction containing a polyhistidine tag (27), was cloned and expressed in Escherichia coli by a modified (see “Acknowledgments”) standard procedures (28) at a yield of 5–10 mg/liter of culture. After protein isolation using a 10-ml nickel-Sepharose fast flow column (Pharmacia Biotech Inc.), the polyhistidine tag was removed with cyanogen bromide (1.5 mg/mg of protein at pH 1), and proinsulin was purified by reverse-phase HPLC (using a Macherey Nagel 300-A C8 column (250 × 10 mm)) with a linear gradient of acetonitrile (22–36% in 30 min) in 0.1% trifluoroacetic acid in water. Proinsulin was labeled biosynthetically with "^15"N by expression of the gene in a culture medium containing "^15"NH4Cl (28, 29), where the incorporation of "^15"N was calculated to be 98.8% by mass spectrometry. Furthermore, proinsulin was double-labeled with both "^2"H and "^15"N by expression in "^15"NH4Cl and 50% "^2"H2O medium, where the final incorporation of "^15"N after protein purification was calculated to be 24.3% (assuming 98.8% "^15"N incorporation). Each proinsulin analogue was cleaved by proteolytic techniques, using trypsin (2.5 μg/mg of protein) and carboxypeptidase B (5 μg/mg of protein) at pH 8 to yield "^14"N- and "^15"N"H-labeled insulin and C-peptide (31- and 33-residue forms). ["^3"H]Phe-"^15"HVal"^14"H insulin was prepared as described previously (30). ["^3"H]Gly, C-peptides ("^15"H-C-peptides) (31- and 33-residue forms) were prepared by total peptide synthesis using standard Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry (Applied Biosystems 430A peptide synthesizer). Subsequent peptide purification was performed by reverse-phase HPLC using similar conditions as described above.

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Extraction of Proteins from Serum

Solid Phase—After dilution with 5 ml of 0.1% trifluoroacetic acid in water, the serum sample (1 ml) was filtered through a 0.45-μm sterile filter (Millipore Corp.), and 150 IU of heparin was then added (to bind Fp, a platelet factor in serum that has a HPLC retention time similar to insulin). A known amount of each internal standard (at a concentration similar to that expected for the target protein) and each carrier (at an excess concentration; 100 nm ["^15"N]insulin, 200 nm ["^15"N]H-C-peptide, and 100 nm ["^15"N]Hproinsulin) were added to the serum. A Sep-Pak cartridge (Waters C18) was washed with pure acetonitrile and then equilibrated in 0.1% trifluoroacetic acid in water. The serum was passed back and forth through the cartridge three times at approximately 1 ml/min using propylene syringes fixed at either end. Two solutions (4 ml) consisting of 20% acetonitrile, 0.1% trifluoroacetic acid in water and 80% acetonitrile, 20% dichloromethane were then washed through the cartridge to remove unwanted proteins (32). The target insulin proteins were co-eluted from the cartridge with 50% acetonitrile, 0.1% trifluoroacetic acid in water (2 ml), and the solution was freeze-dried.

Immunoffinity—After dilution with 5 ml of 0.1 M sodium phosphate buffer, pH 8, the serum sample (1 ml) was filtered through a 0.45-μm sterile filter (Millipore), and 150 IU of heparin and a known amount of each internal standard and each carrier were then added (as for solid phase). The serum was passed twice (under gravity) through an affinity column pre-equilibrated in the phosphate buffer, and the column was then washed with the buffer (6 ml). The column consisted of an Amnolulose gel (3 ml, Pierce) to which both insulin antibodies (guinea pig) and C-peptide antibodies (mouse IgG, known to also bind insulin) were coupled at excess concentration (1 mg/ml). A Sep-Pak cartridge (Waters C18 light) was washed with pure acetonitrile, equilibrated in 0.1% trifluoroacetic acid in water, and fixed on to the affinity column. A solution of 2 μg acetic acid (6 ml) was then passed down the column to elute the target proteins into the Sep-Pak cartridge. The cartridge was then removed and washed with 20% acetonitrile, 0.1% trifluoroacetic acid in water (4 ml). The target insulin proteins were eluted from the cartridge with 50% acetonitrile, 0.1% trifluoroacetic acid in water (2 ml), and the solution was freeze-dried.

Purification of Proteins by Microbore HPLC

Following extraction by either solid phase or immunoffinity, the samples were redissolved in 0.1% trifluoroacetic acid in water (120 μl) and loaded onto a microbore reverse-phase HPLC system (Applied Biosystems 140B, Nucleosil 300-A C18 column (1 × 150 mm)). Each target protein extracted from the serum was then isolated and purified, together with its internal standard and carrier proteins, by elution with a linear gradient of acetonitrile (22–36% in 60 min at a flow of 40 μl/min) in 0.1% trifluoroacetic acid in water. The excess concentration of the carrier allows easy detection and purification of the three proteins of interest, the target proteins, its internal standard, and its carrier, either as a single peak by chromatography. The aliquots collected were freeze-dried.

Quantitation of Proteins by Mass Spectrometry

Aliquots containing each target protein, together with its respective internal standard and carrier proteins, were resuspended in 10 μl of ESI-MS solvent (50% methanol, 49% water, 1% acetic acid). The samples were then analyzed by ESI-MS carried out in positive ionization
Flow of 10 m or (The amount of [2H16]insulin (the internal standard for insulin) added to C-peptide concentration in these samples was also measured by IDA. To compare results obtained by the two techniques, the insulin measurement is the extraction and purification procedure would not affect the ratio. Known concentration), the exact concentration of the target protein in the target protein and the peak height of the internal standard (added at a scan range. By analysis of the ratio between the peak height of the standard, whereas the large carrier signal was designed to be out of this cover the most intense signals of the target protein and its internal standards added were 100 fmol of [2H14]C-peptide 33 and 150–4500 fmol of [2H14]C-peptide 31.

#### Measurement of the 33-Residue C-peptide Levels

The 33-residue form of the C-peptide comprises an additional two amino acids, lysine and arginine, which remain at its C terminus after incomplete cleavage from proinsulin. This form is thought to be present in serum at a far lower concentration than the fully trimmed C-peptide consisting of 31 residues.

The C-peptide concentration of 10 serum samples was determined by CEI. The 33-residue C-peptide and the 31-residue C-peptide were then individually quantitated in these samples by IDA. The amounts of internal standards added were 100 fmol of [3H]C-peptide 33 and 150–4500 fmol of [3H]C-peptide 31.

#### Measurement of Basal Levels in Non-diabetic and Type II Diabetic Individuals

Following a 12-h fast, fresh serum samples were obtained from three types of persons: non-diabetic non-obese (25 samples), non-diabetic obese (10 samples), and type II diabetic (20 samples). A heterogeneous population was examined from both sexes, aged 20–60 years, where obese individuals were defined as those with a body mass index above 35 kg/m². The precise basal level of insulin, C-peptide, and proinsulin in each of these samples was investigated by IDA. The concentration of insulin (in all samples) and C-peptide (only in samples from diabetic individuals) was also measured by immunoassay. The amounts of internal standards added to each sample were 10–300 fmol of [3H]insulin, 200–2000 fmol of [3H]C-peptide, and 10–100 fmol of [15N]proinsulin.

#### Two Post-pancreatic Graft Studies

**Patient 1 (Male, Aged 61)—** This patient underwent a subtotal pancreatectomy with autotransplantation of the islets of Langerhans. Following the operation, insulin and C-peptide basal levels were measured by immunoassay from blood samples (10 ml) taken every 3 h for 60 h. These 20 samples were further analyzed by IDA to determine insulin, C-peptide, and proinsulin levels during this period. After coagulation in the absence of anticoagulant, serum was separated from blood samples by centrifugation. The amounts of internal standards added to each sample were 10–30 fmol of [3H]insulin, 500 fmol of [3H]C-peptide, and 50 fmol of [15N]proinsulin.

**Patient 2 (Female, Aged 52)—** Following a similar operation to that described for patient 1, pancreatic reaction was monitored by an oral glucose tolerance test, where 70 g of glucose in 400 ml of water was taken orally after a 12-h fast. Insulin and C-peptide levels were measured by immunoassay from blood samples (10 ml) taken every 10 min for 50 min. For comparison, insulin, C-peptide, and proinsulin levels concentration was similarly measured by IDA of serum samples at an MEIA insulin concentration of 100, 570, and 890 pm. [15N]Insulin and [15N][14C]C-peptide were added at a concentration similar to that of the target protein (as determined by immunoassay), and [15N]proinsulin (the internal standard for proinsulin) was added at one-tenth the insulin concentration.

![Fig. 1. Purification of proteins by HPLC. Insulin, C-peptide, and proinsulin were separated and purified by microbore HPLC together with their internal standard and carrier proteins after extraction by immunoaffinity from 1 ml of serum. Products were eluted with a linear gradient of acetonitrile (22–36% in 60 min) in 0.1% trifluoroacetic acid in water. The excess concentration of carrier allows clear detection of each target protein.](http://www.jbc.org/fig1.png)
After extraction and purification from serum, the concentrations of insulin, C-peptide, and proinsulin were determined by ESI-MS from the ratio between each target protein and its internal standard (added to 1 ml of serum in known amounts). Horizontal and vertical scales are relative intensity and mass-to-charge ratio, respectively. A, 42 fmol of insulin found relative to 50 fmol of $[^{2}\text{H}_{16}]$insulin added; B, 320 fmol of C-peptide found relative to 500 fmol of $[^{2}\text{H}_{14}]$C-peptide added; and C, 34 fmol of proinsulin found relative to 50 fmol of $[^{15}\text{N}]$proinsulin added. The carrier protein signal is outside the mass scan range (Table I).
Insulin, C-peptide, and Proinsulin Levels in Humans by IDA

RESULTS

Preparation of Standards

Each protein prepared comprises the human sequence and was characterized by amino acid analysis and ESI-MS (Table I). Importantly, all of these analogues prepared, both those to be used as internal standards and those that act as carrier proteins, had exactly the same retention time by HPLC as their native counterparts and differ in mass due only to isotopic labeling. Further, the carrier proteins, present in excess concentration, exhibited no spectral peaks by ESI-MS that might have obscured the measurement of their respective target protein or internal standard peaks.

Extraction and Purification of Proteins from Serum

Insulin, proinsulin, and the C-peptide were efficiently isolated and purified from 1 ml of serum by both solid-phase and immunoaffinity extraction methodology, with approximately 80% recovery of the pure target protein. Both extraction procedures eliminated most of the unwanted substances in the serum (which would have overloaded the mass spectrometer) before further purification by microbore HPLC. This system keeps the working volumes small to avoid protein loss and is relatively efficient even at low protein concentration. Immunoaffinity (Fig. 1) allowed a more specific extraction of the target proteins than solid-phase methodology, which also extracted proteins of similar mass to the C-peptide (such as platelet factors) that elute at an HPLC retention time between that of the C-peptide and insulin.

Following extraction and purification, an excellent signal-to-noise ratio was obtained by ESI-MS of each target protein at its physiological concentration in the presence of its internal standard, demonstrating the high sensitivity of this method (Fig. 2). By extraction of a series of samples containing either negligible amounts of each target protein or no internal standards, we showed that there was no contaminating signal by ESI-MS from endogenous impurities in the serum (remaining after extraction and HPLC purification) that would significantly obscure measurements of the target protein or its internal standard (data not shown).

Verification of IDA Methodology and Comparison to Immunoassay

Serum samples received from the University Hospital, Geneva, were stored at −20 °C for up to 3 months before analysis by IDA. Trial analyses by IDA before and after this period were indistinguishable and showed that there was no significant change in insulin, C-peptide, or proinsulin concentration.

Three sets of four repeat extractions of either insulin, C-peptide, or proinsulin from the same serum sample demonstrated that the variability of results by IDA is below 10% at all concentrations measured. However, we noted that this variability was greater at lower protein concentrations and estimate that the lowest detectable limit within this error margin is at 1 pM. Protein concentration by immunoassay was calibrated using a series of standards supplied by the manufacturer. Analysis by IDA showed that the insulin or C-peptide concentration of each of these standards was in agreement with the concentration of our internal standards, [2H16]insulin and [2H14]C-peptide, to within 10% (data not shown).

The insulin and C-peptide concentration of trial serum samples analyzed by IDA ranged from 3 to 1397 pM (insulin) and from 30 to 5786 pM (C-peptide). Comparison of these results with those by immunoassay shows that measurements are higher by immunoassay for 70% (insulin) and 80% (C-peptide) of the samples (Figs. 3A and 4A). Although the absolute difference between immunoassay and IDA values is larger at higher concentrations, the relative difference is larger at lower concentrations (Figs. 3A and 4A, insets). This may partly be due to larger errors in both techniques at lower concentrations. There is a linear relationship between immunoassay and IDA values with slope 0.88 ± 0.03 (insulin) and 0.82 ± 0.03 (C-peptide) (Figs. 3B and 4B). Using this relationship, theoretical values by IDA were calculated from immunoassay values. A plot of the relative residual between actual and theoretical IDA values (inset) indicates that the bias is not concentration-dependent.

Measurement of the 33-Residue C-peptide Levels

The C-peptide concentration of the 10 samples analyzed ranged from 150 to 4850 pM as determined by CEI. This does not distinguish between the 31- and 33-residue forms of the C-peptide that were shown to react equally well in the immunoassay (data not shown). IDA, however, allows determination of each form of the C-peptide specifically, since the 33-residue C-peptide elutes 2 min after the 31-residue C-peptide by microbore HPLC and they exhibit a distinct difference in mass.
The concentration of the 33-residue C-peptide determined in four of the samples by IDA was 80, 130, 230, and 470 pM, where the 31-residue C-peptide was determined at a concentration of 2100, 2540, 3320, and 4600 pM, respectively. Thus the 33-residue C-peptide concentration in these samples ranged from 4 to 10% of the 31-residue C-peptide concentration. The amount of the 33-residue C-peptide was too low to be measured in the remaining six samples, where the 31-residue C-peptide concentration was less than 1600 pM.

Measurement of Basal Levels

The insulin, C-peptide, and proinsulin concentrations of each sample as determined by IDA were compared with immunoassay results (Figs. 5–7). Insulin and C-peptide basal level values by IDA are at the lower end of the standard values determined by immunoassay (7, 8, 33). Samples from obese individuals show protein levels that were between those from non-diabetic non-obese and type II diabetic individuals. In most cases, insulin and C-peptide levels are higher by immunoassay than by IDA. Interestingly, the difference between MEIA and IDA results is highest for type II diabetic samples, where insulin concentrations are higher (Table II). Although results by IDA show that insulin, C-peptide, and proinsulin levels are all increased in obese and type II diabetic individuals (by 4, 3, and 6 times, respectively), the ratio between proinsulin and insulin levels is increased, whereas the ratio between C-peptide and insulin levels is decreased relative to non-obese non-diabetic individuals (Table II).

Post-pancreatic Graft Studies

Patient 1—The insulin and C-peptide concentration of post-operation samples taken over the 60-h period are slightly lower by IDA than by immunoassay (Fig. 8). Both sets of results remain close to standard basal level values although there is a slight decrease in their concentration over the period. Proinsulin levels are extremely high, fluctuating between 60 and 170 pM (approximately 10 times the normal basal concentration).

Patient 2—Following the administration of glucose to this patient after an operation, insulin and C-peptide levels showed a normal increase to maximum in 20 min followed by a fast decrease to stability after 30 min (Fig. 9). Results by IDA are similar to those by immunoassay although the C-peptide concentration is lower by IDA during the initial 20 min. Proinsulin levels follow a similar trace but increase faster, with a maximum after 10 min.
DISCUSSION

The difficulty in synthesizing analogues of macromolecules has often hindered studies using isotopic dilution methodology. For these investigations, analogues of insulin, C-peptide, and proinsulin were successfully prepared, possibly representing the largest target molecules to have been successfully subjected to the technique of stable isotope dilution mass spectrometry. Neither of the isotopes used in the preparation of these analogues, \(^{15}N\) or \(^{2}H\), are radioactive or harmful in low doses. Thus, each of the isotope-labeled analogues used for initial in vitro work stands as an ideal candidate for possible in vivo work, where exogenous and endogenous protein would be distinguishable by ESI-MS.

The overall protein concentration of serum is too high for it to be feasible to introduce unfractionated samples directly into a mass spectrometer or an HPLC (where even in the presence of a carrier, target proteins are very difficult to detect against the large UV absorbance of serum). A specific extraction methodology is thus required for the quantitation of single proteins. Extraction by immunoaffinity is most specific when the antibody used reacts solely with the target protein. This is often not the case, however, leading to inaccuracies in techniques relying solely on immunological properties. Insulin antibodies used for this study by IDA were shown to bind insulin almost specifically with low C-peptide or proinsulin cross-reactivity, but the C-peptide antibodies bind both C-peptide and proinsulin with equally high affinity. Our method takes advantage of immunological cross-reactivity by using a mixture of antibodies against insulin and C-peptide to extract all three proteins, which are then easily separated and purified by chromatography. Alternatively, solid-phase methodology is far more adaptable to the extraction of many different proteins since antibodies are not required. Although this extraction technique is less specific, the carrier allows clear identification of the target protein from the low quantity of remaining impurities by subsequent chromatography.

Comparison of IDA against standard immunoassay serves to validate our methodology and indicate potential differences. Results were higher by immunoassay than by IDA for many of the samples studied. Differences between the two sets of results cannot be explained purely by experimental error or a difference in the concentration of standards used by each technique. However, the difference between determination of the
C-peptide by CEI (which measures the sum of 31- and 33-residue C-peptide levels indistinguishably) and IDA (where we measure the 31-residue C-peptide levels specifically) may be partially explained by the significant proportion of the 33-residue C-peptide present in serum.

It is now widely accepted that insulin and C-peptide antibodies used in immunoassay techniques can cross-react with proinsulin and its conversion intermediates (4, 5). Although we measured low proinsulin reactivity (not more than 1%) in both MEIA and CEI, these immunoassays were shown to cross-react linearly with 56% (MEIA) and 42% (CEI) of des-31–32 split proinsulin when added in high purity to a series of test samples at known concentrations (from 20 pM to 1 nM) (data not shown).

The preparation of des-31–32 split proinsulin will be described elsewhere.2 Since des-31–32 split proinsulin has been found to be the major conversion intermediate, present at up to 30% of the total insulin-related material in the circulation (4, 7, 34), insulin and C-peptide levels may be overestimated by MEIA and CEI in some cases.

In type II (and pre-type I) diabetic individuals, high levels of proinsulin and its conversion intermediates have been previously found relative to insulin (4, 13, 23–26, 35). However, this is only a relative measurement. Most conventional techniques cannot measure the absolute change in concentration of each protein specifically. Our studies indicate that, although insulin levels are increased in these individuals, abnormality in the ratio of circulating components is primarily caused by a large increase in proinsulin levels (Table II). This may be due to overstimulation of the pancreatic beta cells beyond their capacity to complete the proinsulin-to-insulin processing (possibly caused by insulin resistance or decreased glucose tolerance) or an intrinsic defect in expression or structure of the conversion enzymes (36, 37).

Studies using IRMA (a monoclonal antibody-based two-site immunoradiometric assay) suggest that both proinsulin and des-31–32 split proinsulin levels increase in type II diabetic subjects (4, 7, 8). Although IRMA is one of the most specific immunoassays to date, the proinsulin conversion intermediates cannot yet be fully distinguished. Since IDA does not require antibodies of high specificity, we feel that the individual measurement of each conversion intermediate would be a promising area of research using this new methodology.

A disproportional increase in the levels of des-31–32 split proinsulin would increase the proportion of cross-reactivity measured by MEIA. Our results, therefore, may confirm studies by IRMA since we see a larger difference between MEIA readings and the more specific IDA readings of insulin in samples from type II diabetic subjects (Table II). Notably, the ratio between MEIA and IDA values for insulin levels in non-diabetic individuals was calculated to be 1.3 (Table II). This is in good agreement with our preliminary comparison of the two techniques by measurement of insulin levels in samples taken from various types of individuals who were mainly non-diabetic, where the ratio between MEIA and IDA results was 1.2 (Fig. 3B).

Results by IDA indicate that C-peptide levels increase in obese and type II diabetic individuals to a lower extent than insulin levels do, resulting in a significant decrease in the ratio between them (Table II). The exact role of the C-peptide is not

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### Table II

<table>
<thead>
<tr>
<th>Protein</th>
<th>Technique</th>
<th>Mean concentration from samples measured&lt;sup&gt;a&lt;/sup&gt; (pm)</th>
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<td></td>
<td>Non-obese (see Fig. 5) Obese (see Fig. 6) Type II diabetic (see Fig. 7)</td>
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<td>Insulin</td>
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<tr>
<td>Proinsulin</td>
<td>IDA</td>
<td>6 (±1) 89 (±26) 821 (±24)</td>
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<td>C-peptide</td>
<td>CEI</td>
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<td>C-peptide&lt;sup&gt;β&lt;/sup&gt; insulin</td>
<td>IDA</td>
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</table>

<sup>a</sup> Errors quoted are standard errors of the mean.

<sup>b</sup> Mean ratio of those determined from each sample.

<sup>c</sup> Data not determined.

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FIG. 8. Post-pancreatic graft study, patient 1. Insulin, C-peptide, and proinsulin basal levels were determined by IDA in serum samples taken every 3 h for 60 h after the operation. Results were compared with insulin measurements by MEIA and C-peptide measurements by CEI.
clearly understood although it exhibits specific biological activity of its own, with a partial effect on glucose transport, blood flow, capillary diffusion capacity, glucose utilization, and renal function in type I diabetic patients (38, 39). Insulin and the C-peptide are secreted in an equimolar ratio after the enzymatic breakdown of proinsulin. Since insulin is prone to a variable hepatic extraction and peripheral clearance (40, 41), C-peptide levels are frequently used to measure insulin secretion and beta cell function. However, this measure may not be accurate since our studies suggest that the ratio between insulin and C-peptide levels is variable between different types of individuals (possibly due to variability in the rate of elimination of both insulin and C-peptide from the circulation).

The post-pancreatic graft studies represent examples of a clinical situation where the accurate measurement of protein levels is of critical importance in assessing the success of the operation. Results obtained by IDA compare well with immunoassay data. The high and unstable level of proinsulin measured in patient 1 immediately after the operation was thought to be due to initial overcompensation of the secretory activity of the pancreatic beta cells following a decrease in their mass after autotransplantation. Several days later, proinsulin levels returned to normal (data not shown). This phenomenon was not detected before investigations by IDA, since insulin and C-peptide levels are relatively normal. The slow decrease in insulin and C-peptide concentration may simply be restabilization of the pancreas. Patient 2 showed a normal response to glucose, but the high initial increase in proinsulin levels detected by IDA could have resulted from overcompensation of the beta cells as discussed for patient 1.

We conclude that, in general, results between immunoassay and IDA correlate well. However, where conventional immunoassays (such as routinely used forms of radioimmunoassay and MEIA) are perturbed by such factors as very high or very low concentration levels (where calibration strays from linearity) or the presence of extraneous cross-reacting material (the magnitude of which cannot be calculated), much larger deviations are seen. Significant differences between the two sets of results outline the need of a definitive technique that avoids the problems associated with present methodology, thus increasing the accuracy of important clinical results and their interpretation.

Specifically, these studies demonstrate how IDA could be used toward understanding the behavior of insulin in humans. A precise, simultaneous measurement of all insulin-related proteins in blood, including proinsulin conversion intermediates and insulin fragments, would help answer many of the questions relating to diabetes and become a valuable diagnostic and prognostic test in itself. Abnormal ratios between insulin and proinsulin-related proteins may, for example, be a good index of a pancreatic transplant on the verge of failing. Current methods, when the whole organ is involved, are based on determination of amylase in urine. Even this rather indirect method is not possible when the islets alone are transplanted.

Improvement in the accuracy of measurement of protein levels in humans is an important step toward a better understanding of many common and serious diseases. IDA is a conceptually simple and adaptable technique that could be used to study many different metabolic or physiological processes. Although for clinical analyses IDA is not as routine a method as standard immunoassay since measurements take longer to perform and special equipment is required, it could provide additional insight into difficult or suspect cases. Continual improvements in protein labeling facilities and mass spectrometric instrumentation could allow IDA to become a powerful tool for general studies on protein behavior.

Acknowledgments—We thank Drs. Alain Golay and Yves Morel for providing clinical serum samples, Dr. Jan Markussen of Novo Nordisk, Denmark, for providing antiserum against human C-peptide (mouse IgG1), Prof. Philippe Halban and Yves Brandenburger for important discussions, Drs. Reto Stöcklin, Jean-François Arrighi, and Kahn Hoang-Van Pol Wolk on the modification of the proinsulin expression discussions, Drs. Reto Stöcklin, Jean-François Arrighi, and Kahn Hoang-Van Pol Wolk on the modification of the proinsulin expression outline the need of a definitive technique that avoids the problems associated with present methodology, thus increasing the accuracy of important clinical results and their interpretation.

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FIG. 9. Post-pancreatic graft study, patient 2. Insulin, C-peptide, and proinsulin levels were determined by IDA in serum samples taken every 10 min for 50 min after 70 g of glucose in 400 ml of water was taken orally after a 12-h fast following the operation. Results were compared with insulin measurements by MEIA and C-peptide measurements by CEI.
Insulin, C-peptide, and Proinsulin Levels in Humans by IDA


Development of an Isotope Dilution Assay for Precise Determination of Insulin, C-peptide, and Proinsulin Levels in Non-diabetic and Type II Diabetic Individuals with Comparison to Immunoassay
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doi: 10.1074/jbc.272.19.12513

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