

Human Serum Proinsulin

F. MELANI, A. H. RUBENSTEIN, and D. F. STEINER

From the Departments of Biochemistry and Medicine, University of Chicago, Pritzker School of Medicine, Chicago, Illinois 60637

ABSTRACT Gel filtration of human serum extracts on Bio-Gel P-30 columns produced two peaks of material reactive with insulin antisera. The earlier eluting fraction appeared at the elution position of proinsulin (serum proinsulin-like component, PLC) while the second fraction corresponded in elution volume to insulin. In assays using porcine insulin-¹²⁵I and an antiserum against porcine insulin, human pancreatic proinsulin was less reactive than human insulin. Serial dilutions of the serum PLC in the immunoassay showed immunological identity with the human proinsulin standard. Partial tryptic digestion of the serum PLC yielded products with increased immunological reactivity as estimated with insulin as the standard. With larger amounts of trypsin, all the serum PLC was converted to insulin-like components (desthreonine and desoctapeptide insulin). On the basis of these results we conclude that the earlier eluting fraction of human serum extracts is proinsulin.

The fasting values of proinsulin in normal subjects ranged between 0.05 and 0.4 ng/ml, representing from 5 to 48% of the insulin concentration. In one subject the values of proinsulin were higher than those of insulin. After oral administration of 100 g of glucose, the proinsulin levels tended to rise similarly to insulin. Three obese patients with hyperinsulinemia had higher fasting levels of proinsulin and a greater increase after glucose than the normal subjects. As the high levels of proinsulin coexisted with raised insulin concentration in these obese subjects, the relative proportions of the two hormones were in the same range observed in the normal group. Thus hyperinsulinemia in these obese subjects was not accompanied by an increase in the fraction of serum proinsulin. When the values for serum proinsulin were expressed as percentage of the insulin levels,

there was a decrease in the per cent proinsulin in the first hour of the glucose tolerance test. After the second hour, the per cent tended to rise towards the fasting levels.

INTRODUCTION

The recent discovery of proinsulin, a single chain precursor of insulin (1), has led to the successful isolation and structural elucidation of this protein in several mammalian species (2, 3).¹ The single polypeptide chain of proinsulin begins at the amino-terminus with the B chain of insulin, proceeds through a connecting polypeptide segment of about 30 amino acids, and terminates with the A chain (1, 2). The characteristic disulfide bonds of insulin are present in this molecule,² and various physical and immunologic data indicate that the spatial arrangement of the insulin portion of the molecule is closely similar, if not identical, to that of insulin itself (3-6). Some structural elements of the connecting polypeptide portion of proinsulin have been shown to be capable of eliciting specific antibodies when guinea pigs are immunized with bovine proinsulin (6). Thus, in general, antisera of proinsulin react more strongly with proinsulin than with insulin, and the reverse situation occurs with insulin antisera (3). Moreover, considerable immunological differences have been demonstrated between human, porcine, and bovine proinsulins (6). These are in accord with the relatively large degree of variability in the amino acid sequences of the connecting polypeptide segments of bovine, porcine, and human proinsulin (7). Thus antisera against human proinsulin will be needed for the specific immunological detection of human proinsulin. However, until sufficient supplies of human proinsulin can be obtained for immunization of animals, it can be measured after separation from insulin by means of its cross-reactivity in the ordinary in-

Dr. Melani is an Eli Lilly Research Fellow, 1968-69. His permanent address is the Department of Endocrinology, University of Ulm, West Germany.

This work was presented in part at the 29th Annual Meeting of the American Diabetes Association, June 1969.

Received for publication 8 September 1969 and in revised form 27 October 1969.

¹ Nolan, C., E. Margoliash, and D. F. Steiner. 1969. Manuscript in preparation.

² Birdwell, C., and D. F. Steiner. Unpublished results.

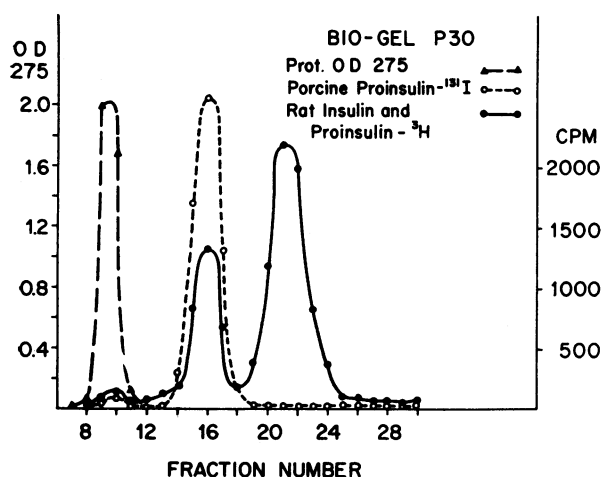


FIGURE 1 Separation of a mixture of labeled insulin and proinsulin on 1 × 50 cm column of Bio-Gel P-30 equilibrated in 3 M acetic acid. Each fraction was counted in an autogamma and a liquid scintillation spectrometer.

ulin immunoassay system standardized with human proinsulin and insulin.

In a preliminary report, evidence was presented indicating that material having some physical and immunologic characteristics of proinsulin is present in human plasma and urine samples (8). We have now characterized the circulating proinsulin-like material in greater detail by showing its immunologic identity with authentic human proinsulin separated in nearly pure form from crystalline human insulin and by studies of its trans-

formation to insulin-like components by controlled tryptic digestion. Some observations on the levels of proinsulin during 4-hr oral glucose tolerance tests in normal subjects and in obese subjects with hyperinsulinemia are also included in this report.

METHODS

Human and porcine insulin (25 U/mg) were kindly supplied by the Novo Company, Copenhagen, and were both prepared by standard commercial procedures; purified porcine proinsulin was a gift from Dr. Ronald E. Chance of The Lilly Research Laboratories, Indianapolis. Human proinsulin was separated from the crystals of human insulin by gel filtration on Sephadex G-50 in 1 M acetic acid in Copenhagen and was further purified by chromatography on diethylaminoethyl (DEAE) cellulose in our laboratory by methods similar to those described previously for the preparation of bovine proinsulin (3). It consisted of material which on polyacrylamide gel electrophoresis (7.5%, pH 8.9) gave a major band corresponding to labeled human proinsulin derived from incubated islet cell adenoma slices (5). Amino-terminal analysis by the dansyl method (9) yielded a large quantity of dansyl-phenylalanine as expected for a single chain proinsulin and only traces of dansyl-glycine. Amino acid analysis indicated the characteristic composition of insulin with increased amounts of those amino acids present in the connecting peptides of bovine, porcine, and rat proinsulin.

As the quantity of human proinsulin was limited, the protein was labeled with ¹²⁵I for further characterization. After iodination, the human proinsulin-¹²⁵I was purified on a cellulose column from which it was eluted with plasma (10). The human proinsulin-¹²⁵I was subsequently separated from the plasma protein by gel filtration on a 1 × 50 cm column of Sephadex G-50 in 1 M acetic acid. After evaporating the acetic acid to dryness, a portion of the labeled proinsulin was dissolved in 200 μl of 0.01 M Tris (hydroxy-

TABLE I
Clinical Data and Blood Sugar (BS) (mg/100 ml) and Immunoreactive Insulin (IRI)*
(μU/ml) after Administration of 100 g of Oral Glucose

Subjects	Sex	Age	Ideal BW‡	F		½ hr		1 hr		2 hr		3 hr		4 hr	
				BS	IRI	BS	IRI	BS	IRI	BS	IRI	BS	IRI	BS	IRI
			yr	%											
Normal															
A. H.	M	25	−3	84	10	96	24	118	25	104	20	68	16	70	19
C. B.	M	22	−6	70	30	126	80	106	60	—	—	81	30	67	10
F. M.	M	39	+3	85	20	92	44	102	60	82	32	78	28	80	22
P. O.	M	25	−3.5	82	10	178	70	157	135	95	70	64	20	80	10
J. C.	M	28	−2.5	90	10	147	60	179	100	117	88	—	—	103	40
L. C.	M	22	−8	93	28	182	108	159	94	109	68	97	54	50	36
Obese															
H. J.	F	38	+90	76	38	114	130	202	150	164	180	130	90	70	35
G. N.	F	24	+146	86	36	220	160	228	110	226	260	154	240	104	86
M. S.	F	16	+223	76	160	140	640	216	1040	282	1100	191	1040	85	280

* The serum insulin was measured against a human insulin standard using a tracer of porcine insulin-¹²⁵I and a porcine insulin antiserum.

† Metropolitan Life Insurance Tables.

methyl) aminomethane/hydrochloric acid, 0.05 M sodium chloride (pH 7.7) and incubated with 0.05 μ g of trypsin for 30 min at 37°C. 1 mg of cold human insulin in 0.8 ml of 1 M acetic acid was added to the mixture; and after gel filtration (1 \times 50 cm G-50 Sephadex column), one peak of radioactivity corresponding in position with the optical density of the carrier insulin was observed. The tubes containing the radioactivity were combined and evaporated to dryness in vacuo. Thereafter the trypsin-converted labeled material was sulfitolyzed (11) and subjected to paper electrophoresis on Whatman 3 MM paper in 8 M urea 20% acetic acid solution (pH 3.2) for 16 hr at 150 v/cm. Two radioactive peaks were observed corresponding in mobility to the S-sulpho A and B chains of the unlabeled marker human insulin. In contrast the sulfitolyzed human proinsulin- 125 I migrated as a single component after electrophoresis, indicating that it consisted of a single polypeptide chain.

Gel filtration of serum extracts. Proinsulin and insulin were separated on 1 \times 50 cm column of Bio-Gel (Bio-rad Labs, Richmond, Calif.) (P-30, 100-200 mesh) equilibrated with 3 M acetic acid. The fraction size was 1.2-1.3 ml and the void volume of the column, determined by the optical density of BSA (bovine serum albumin) was 12-13 ml. The columns were calibrated with a mixture of porcine proinsulin- 125 I and rat leucine-insulin- 3 H or porcine insulin- 125 I (Fig. 1). In all samples recovery of immunoreactive insulin in the column fractions was greater than 70% of the values determined by direct immunoassay of serum. To minimize the adsorption of insulin or proinsulin the glass tubes were coated with an aqueous solution of 1% silicone (Siliclad, Clay-Adams, Inc., New York).

Iodination with 125 I. Porcine insulin and proinsulin and human proinsulin were labeled using the method of Hunter and Greenwood (12) and purified on 0.5 \times 5 cm cellulose columns (10).

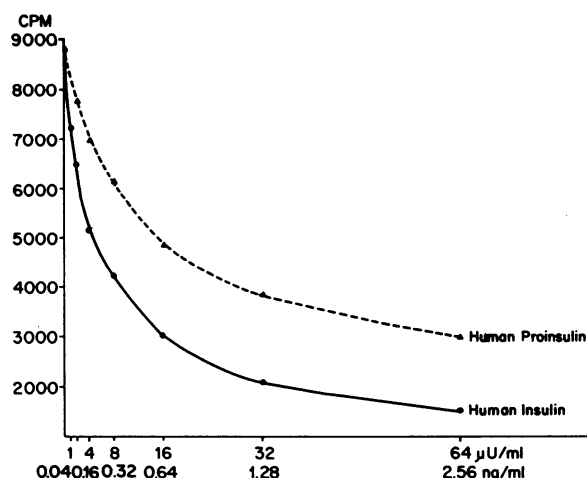


FIGURE 2 Porcine insulin- 125 I, pork insulin anti-serum. Standard curves of human proinsulin and human insulin using porcine insulin- 125 I and anti-porcine insulin serum. The cpm on the ordinate represents the radioactivity in the precipitate corresponding to antibody bound porcine insulin- 125 I.

Immunoassay. A modification of the double antibody method of Morgan and Lazarow (13) was used; Tris-albumin buffer, pH 7.7 (0.25% BSA, 0.1 M Tris (hydroxymethyl) aminomethane/hydrochloric acid, 0.05 M sodium chloride) was the diluent and the reactions were carried out as follows: 1st reaction (48 hr): to 1 ml of buffer containing human insulin or human proinsulin standard or diluted sample, 0.1 ml porcine insulin- 125 I (0.05-0.1 ng) and 0.1 ml guinea pig anti-porcine insulin serum (final dilution 1:120,000) were added and mixed. 2nd reaction (48 hr):

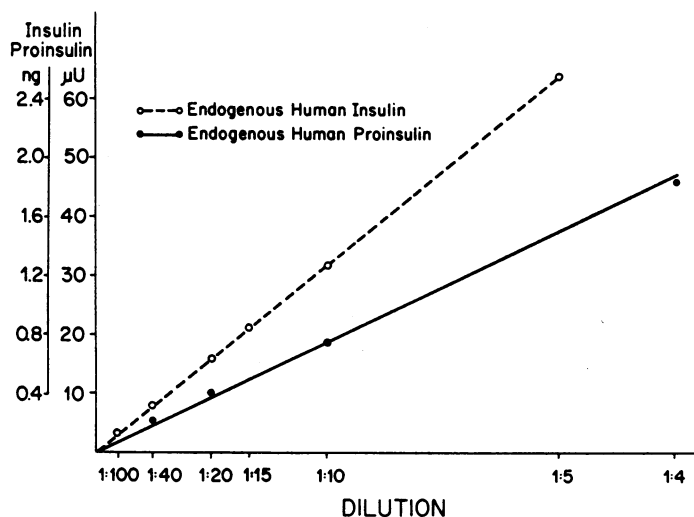


FIGURE 3 Porcine insulin- 125 I, pork insulin anti-serum. The effect of dilutions of serum proinsulin and insulin on the measured concentrations of these hormones. The insulin and proinsulin were obtained after gel filtration of pooled serum extracts from a normal subject after oral administration of 100 g of glucose. The values of insulin and proinsulin were derived from the corresponding standard curves.

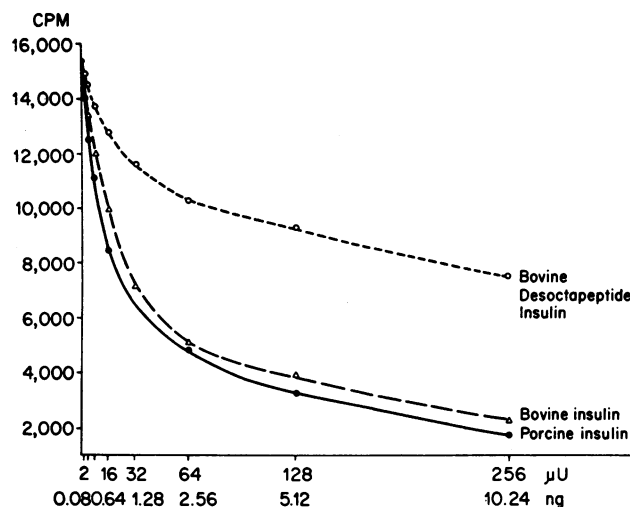


FIGURE 4 Standard curves of desoctapeptide bovine insulin and bovine and porcine insulin using antibody against porcine insulin and porcine insulin- ^{125}I . The desoctapeptide insulin displaces the tracer much less efficiently than the intact insulin on a weight basis. Similar results would be expected for desoctapeptide porcine or human insulin which are identical in structure.

to the above system 0.1 ml of normal guinea pig (1:400) and 0.1 ml of rabbit anti-guinea pig globulin serum (Sylvana Chemical Company, Orange, N. J.) were added and mixed. After centrifugation at 3,000 rpm for 30 min, the supernatant was decanted and the precipitates counted in a Packard autogamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Serum extraction. Aliquots of fresh or frozen serum (7–10 ml) were extracted with acid-alcohol using a modification of the method of Davoren (14). 2 ml of water and 7.5 ml of acid-alcohol (375 ml of 95% alcohol, 7.5 ml of concentrated hydrochloric acid) were added to each milliliter of serum and allowed to stand at 4°C for 10 hr. After centrifugation, the pH of the supernatant was adjusted to pH 8.5 with concentrated ammonium hydroxide. The precipitate was removed by centrifugation and the supernatant adjusted to pH 5.3 with hydrochloric acid (using methyl red as the indicator). After adding 0.025 ml of 2 N ammonium acetate (pH 5.3) for each milliliter of extract, the proteins were precipitated with 15 ml of absolute alcohol and 25 ml of diethyl ether/10 ml of supernatant. After 12–16 hr at 4°C, the samples were centrifuged and the precipitate dissolved in 3 M acetic acid. All glassware used during the extraction procedure was coated with an aqueous solution of 1% silicone.

Trypsin conversion. The buffer used in these experiments was 0.1 M Tris-HCl (pH 7.7). The conversion of porcine proinsulin, which was used because of an insufficiency of the human material, was carried out as follows: 5 ng of proinsulin and 0.1 ng of porcine proinsulin- ^{125}I (approximately 120,000 cpm) were dissolved in 0.9 ml of buffer and placed in a water bath at 37°C. 0.5 μg (0.1 ml) TPCK-trypsin³ (prepared by the method of Wang and Carpenter) (15) was added and 0.2 ml of the incubation mixture was

removed after 5 and 10 min. In order to prevent further tryptic action, 0.2 ml of Trasylol (5000 kallikrein inactivator units (KIU)/ml; FBA Pharmaceuticals, Inc., New York) and 0.4 ml of acetic acid containing 2.5 mg of BSA was added to each sample.

The conversion of human serum proinsulin-like component (PLC) was carried out by adding 0.5 μg of trypsin to 3 ng of serum PLC estimated on the insulin standard (total volume = 0.5 ml). After 5 min at 37°C, 0.5 ml of 6 M acetic acid, containing 2.5 mg of BSA, was added to the solution which was then fractionated on a P-30 column as described above. In several additional experiments, serum PLC was incubated at 37°C with 1 μg of trypsin in 0.5 ml of Tris buffer for 5 min. After addition of 0.3 ml of Trasylol, the solution was diluted with Tris-albumin buffer for direct immunoassay.

Blood sugar. This was measured using the potassium ferricyanide method on the Technicon AutoAnalyzer (Technicon Instruments Corporation, Ardsley, N. Y.) (16).

Patients. Six healthy volunteers of normal weight and three markedly obese subjects with hyperinsulinemia were studied during a 100 g oral glucose tolerance test (Table I). All subjects were instructed to ingest a high carbohydrate diet for 3 days preceding the test which began at 8 a.m. after an overnight fast. One normal subject (P. O.) gave a history of diabetes in a maternal grandmother, while patient M. S. had a paternal uncle with diabetes.

RESULTS

The resolution achieved with Bio-Gel P-30 columns was superior to that obtained previously using Sephadex G-50 (8). Using porcine insulin- ^{125}I and an antiserum against porcine insulin, human proinsulin was less efficient (either on a weight or molar basis) in displacing the tracer from the antibody than was human insulin.

³ TPCK = L-1-tosylamido-2-phenylethyl chloromethyl ketone.

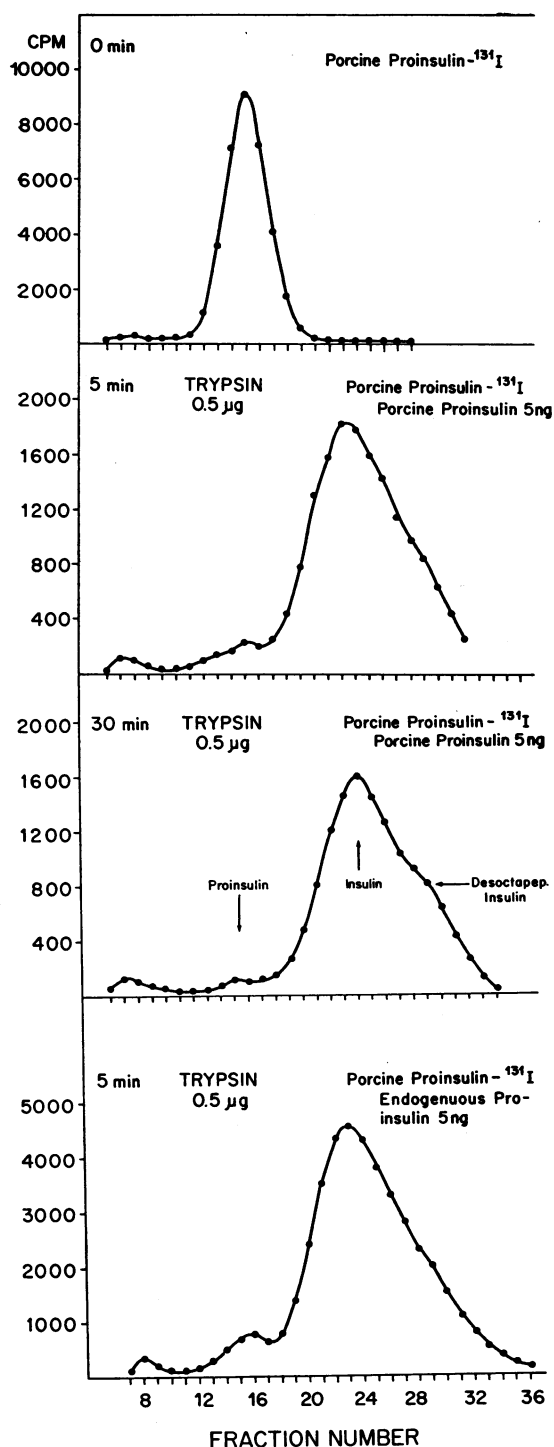


FIGURE 5 Tryptic conversion of a small amount of porcine proinsulin using porcine proinsulin- ^{125}I as a tracer. Proinsulin and insulin were separated on a polyacrylamide column (1×50 cm) equilibrated in 3 M acetic acid. In the upper panel the elution pattern of porcine proinsulin- ^{125}I is shown. With $0.5 \mu\text{g}$ of trypsin (for 5 min) more than 90%

(Fig. 2). In order to use this radioimmunoassay system to measure the concentration of proinsulin, the immunological relationship to the serum proinsulin-like component (PLC) was first compared with authentic human pancreatic proinsulin. For this purpose 50 ml of serum, obtained from a normal patient after glucose administration, was extracted with acid-ethanol and the serum PLC separated on polyacrylamide columns. The PLC peaks were then combined and assayed in several dilutions against the proinsulin standard. When the dilutions were plotted on the abscissa and the measured concentrations on the ordinate, the values fell along a diagonal line (Fig. 3). Such a relationship indicates that the material of the earlier peak is identical in immunological reactivity with human proinsulin. Similar results were obtained when the serum insulin, separated from serum extracts by gel filtration, was measured against the standard of human pancreatic insulin (Fig. 3).

The serum PLC was further characterized by incubation with trypsin. When porcine or bovine proinsulin is incubated with trypsin, dealanated insulin is liberated together with a large part of the connecting segment. Essentially identical results were obtained with human proinsulin in an earlier study (1). If an excess of trypsin is used or the incubation is prolonged, a greater proportion of desoctapeptide insulin, which reacts less well in immunoassays than dealanated or intact insulin (17), is formed (Fig. 4). To determine the conditions for conversion of a small quantity of serum PLC, the required concentration of trypsin was determined in preliminary experiments using porcine proinsulin as the substrate. Fig. 5 illustrates the conversion of 5 ng of porcine proinsulin to an insulin-like component, as shown by the tracer of porcine proinsulin- ^{125}I added to monitor the reaction. After incubation for 5 min with $0.5 \mu\text{g}$ of trypsin, essentially all the proinsulin was converted to dealanated and some desoctapeptide insulin. Prolongation of the incubation to 30 min yielded a greater proportion of desoctapeptide insulin. Substitution of 5 ng of the serum PLC in place of the unlabeled porcine proinsulin in this system also resulted in the conversion of more than 90% of the labeled material to a mixture of dealanated insulin and small amounts of the desoctapeptide form (lower panel of Fig. 5). On the basis of these results this experiment was repeated using the immunoassay to characterize the tryptic conversion products

of a 5 ng porcine proinsulin is converted to an insulin-like component as detected by counting the radioactivity of proinsulin- ^{125}I used as a tracer to monitor this reaction (second panel). After longer incubation with trypsin (30 min), a part of the radioactivity elutes in the region of desoctapeptide insulin (third panel). Substitution of porcine proinsulin with the same amount of human serum PLC (proinsulin-like component) results in a similar degree of conversion (lower panel).

of serum PLC (Fig. 6). As proinsulin is underestimated against the insulin standard (see Fig. 2), conversion of human proinsulin to an insulin-like component (desthreonine insulin in this case) should result in an increase in its apparent immunological reactivity with this standard. In fact, incubation of 3 ng of serum PLC (as measured from the insulin standard) with trypsin yielded 1 ng of immunological activity in the proinsulin region and 3.5 ng in the insulin region after column separation. Thus the expected increase of total immunological activity was obtained. Using a large excess of trypsin (100 μ g for 5 min) essentially all the serum PLC (4.5 ng as measured from the insulin standard) was converted to dealanated and desoctapeptide insulin (lower panel of Fig. 6). This increase in immunological activity was substantiated by direct immunoassay of PLC before and after tryptic conversion in four additional experiments. The increase of immunological activity ranged between 1.8- and 3.0-fold depending on the extent of conversion in each experiment. These results support the conclusion that all the material eluted in the earlier peak is proinsulin or closely related intermediate forms of proinsulin (3).

The clinical data on the subjects and their blood sugar and serum insulin (IRI) are summarized in Table I. Fig. 7 shows the concentration of insulin and proinsulin in one of the healthy subjects after administration of oral glucose. The absolute proinsulin concentration rises after glucose in a fashion similar to insulin. When expressed as a percentage of the insulin concentration, the proinsulin values tend to rise late in the test. The results obtained in the six normal subjects are shown in Fig. 8. The proinsulin concentration rises slightly after glucose and returns to fasting values at the end of the test. Fig. 9 illustrates the results obtained in a markedly obese patient. The absolute values of proinsulin in this subject are very high, but are similar to those of normal subjects when expressed in per cent of the insulin concentration. A summary of the results obtained in the three obese subjects is shown in Fig. 10. Although the absolute values vary, the patterns are similar in all the patients. There is an increase of proinsulin after glucose which parallels the increase of insulin. The values of proinsulin in normal and in obese subjects, expressed as percentage of the insulin levels, are summarized in Table II. Initially there is a decrease in the per cent of proinsulin because of the relatively greater rise in insulin, while towards the end of the glucose tolerance test the per cent again tends to increase towards the fasting level.

DISCUSSION

Using porcine insulin-¹²⁵I and an antiserum against porcine insulin in the immunoassay, the standard curves

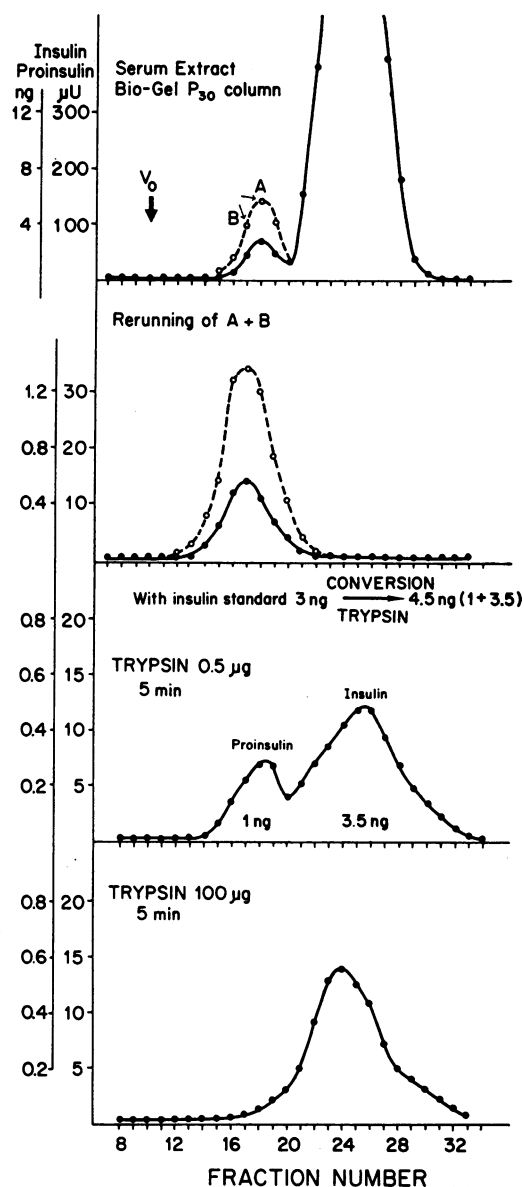


FIGURE 6 Tryptic conversion of human serum PLC to an insulin-like component. PLC was extracted and separated by gel filtration from serum of an obese subject (upper panel). Tubes A and B did not contain insulin as shown by rerunning an aliquot on the column (second panel). The dashed line represents values determined from the human proinsulin standard and the continuous line values determined from the human insulin standard. The third panel shows the conversion of 3 ng of serum PLC using the immunoassay to measure the concentrations of the immunologically reacting material found in each fraction after gel filtration. When incubated with a much larger amount of trypsin, all the immunoreactive material was transformed to insulin-like material (lower panel). In this experiment the amount of desoctapeptide insulin formed (fractions 26-32) was markedly underestimated as the values were all determined from the standard curve for intact human insulin (see Fig. 4).

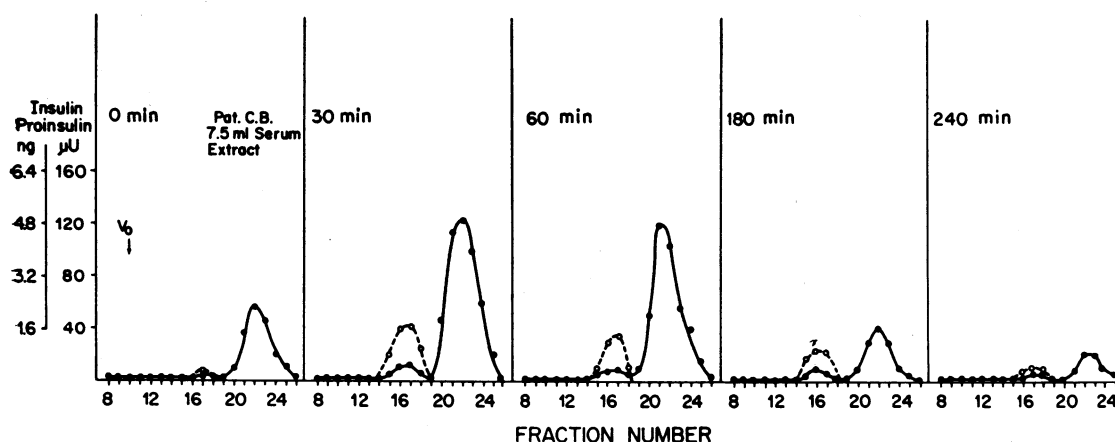


FIGURE 7 Column separation by gel filtration of serum insulin and proinsulin in a normal subject after oral administration of 100 g of glucose. The dashed line represents the immunoassay values estimated from the human proinsulin standard and the continuous line represents the values estimated from the human insulin standard.

of human proinsulin and insulin had different slopes when expressed either on a molar or weight basis. Human proinsulin reacted less efficiently than human insulin in this system and therefore human proinsulin

would be underestimated if read against the insulin standard. When the material of the earlier peak, derived by gel filtration of serum extracts, was measured in several dilutions, it showed immunological identity with

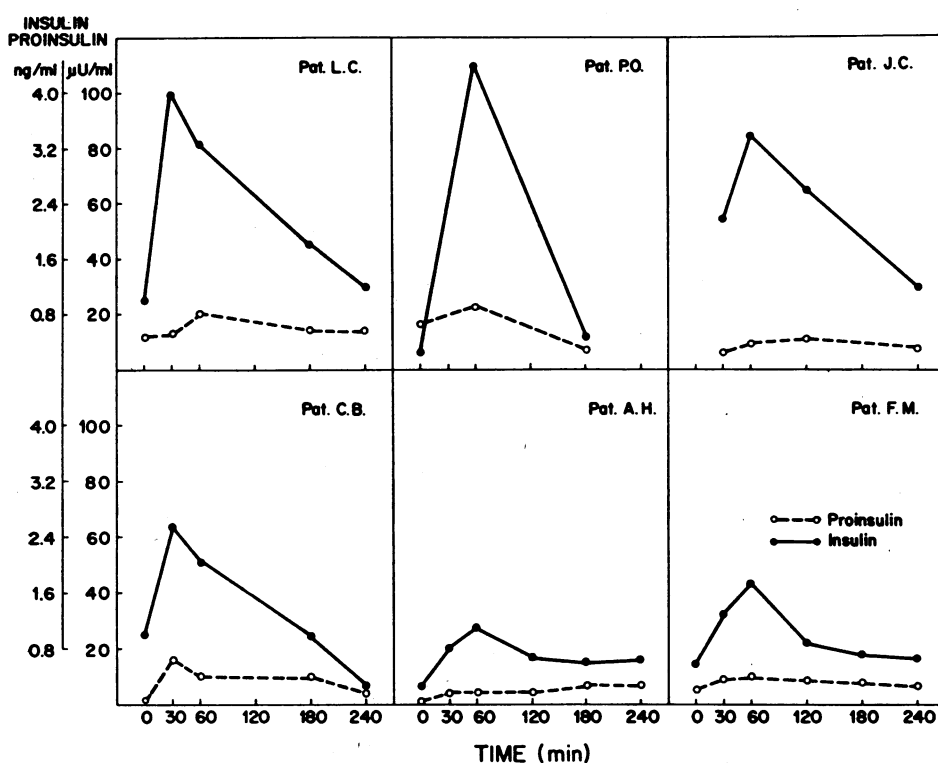


FIGURE 8 Absolute concentration of serum proinsulin and insulin in six normal subjects after oral administration of 100 g of glucose. Porcine insulin-¹²⁵I and porcine insulin antiserum were used for immunoassay. The insulin values were read from a standard of human insulin and the proinsulin values from a standard of human proinsulin.

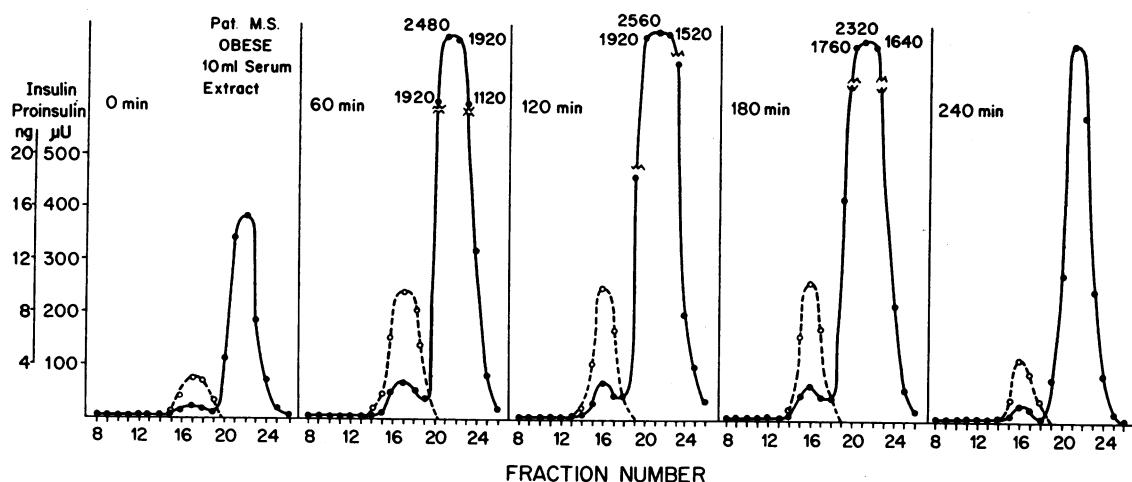


FIGURE 9 Column separation by gel filtration of insulin and proinsulin in an obese subject after oral administration of 100 g of glucose. The dashed line represents the values estimated from the human proinsulin standard and the continuous line the values estimated from the human insulin standard.

purified human pancreatic proinsulin. Therefore the values read from the proinsulin standard should be correct.

The results obtained with trypsin conversion of serum PLC also support the validity of the assay system used and provide further evidence that the serum component is proinsulin. Trypsin rapidly cleaves bovine (or porcine) proinsulin at the arginine-glycine bond (residues 60–61) linking the A chain to the connecting seg-

ment, and at the lysine-alanine bond (residue 29–30) of the B chain, as well as between residues 31 and 33 in the connecting segment. These cleavages result in the liberation of dealanated insulin, the connecting peptide, and alanyl-arginine. With a larger amount of trypsin, a further cleavage at the arginine-glycine bond (residue 22–23) of the B chain produces desoctapeptide insulin which is immunologically less active than intact insulin. Because human proinsulin is underestimated in

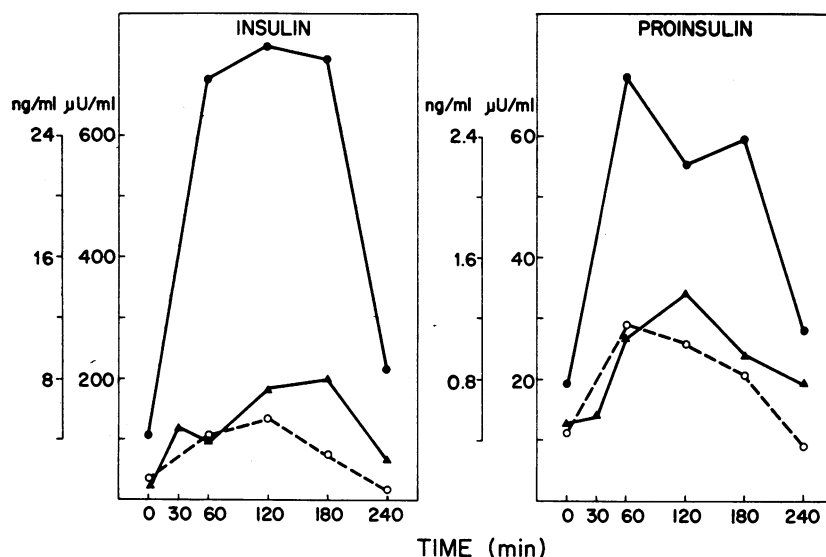


FIGURE 10 Absolute concentrations of insulin (left panel) and proinsulin (right panel) in three obese subjects after oral administration of 100 g of glucose. Porcine insulin- ^{125}I and porcine insulin antiserum were used for immunoassay. The insulin values were read from a standard of human insulin and the proinsulin values from a standard of human proinsulin.

TABLE II

*Proinsulin Values of Normal and Obese Subjects in the Fasting State and after Oral Glucose Administration. The Values, Expressed as Percentage of the Insulin Peak Obtained by Gel Filtration of Serum Extracts, Were Estimated from the Insulin (Ins) and Proinsulin (Pro) Standard**

Subjects	F		$\frac{1}{2}$ hr		1 hr		2 hr		3 hr		4 hr	
	Ins	Pro	Ins	Pro	Ins	Pro	Ins	Pro	Ins	Pro	Ins	Pro
Normal												
A. H.	8	32	6	19	5	14	7	20	23	50	16	45
C. B.	2	6	6	22	5	19	—	—	14	36	29	69
F. M.	13	40	9	27	7	23	13	40	24	50	16	40
P. O.	79	278	—	—	6	21	—	—	18	54	—	—
J. C.	—	—	4	11	3	11	5	17	—	—	7	26
L. C.	18	48	5	13	10	25	—	—	11	30	15	44
Obese												
H. J.	9	37	—	—	7	25	8	22	9	32	13	45
G. N.	11	40	4	12	10	32	5	18	4	13	7	26
M. S.	5	17	—	—	3	10	2	7	2	8	3	13

* Porcine insulin-¹²⁵I and porcine insulin antiserum were used in the immunossay.

terms of the insulin standard in our assay system, its conversion to desethionine insulin should theoretically result in an increased insulin value when read against this standard. After incubation of a sample of serum proinsulin with a low concentration of trypsin, we observed an increase in immunological activity. Despite conversion of only about 70% of the proinsulin (3 ng), 4.5 ng was found after the digestion, representing an increase of 50% in terms of the insulin standard. This increase in immunological activity was confirmed by direct immunoassay of PLC before and after trypsin conversion in several additional samples.

The earlier peak after gel filtration of commercial crystalline insulin contains not only proinsulin but intermediate and nonconvertible (an insulin aggregate unrelated to proinsulin) fractions as well. Although proinsulin and the related intermediate form can be converted to dealanated insulin by trypsin, the nonconvertible fraction is unchanged as judged by gel filtration even when large quantities of the enzyme are used. For this reason it was important to determine whether all the earlier eluting material in serum could be converted with trypsin. When large amounts of trypsin were used, essentially all the immunoreactive material appeared in the insulin containing fractions. This observation is in keeping with the finding that the nonconvertible material has not been identified in biosynthetic studies using rat islets in vitro (6) but has only been found after purification of pancreatic insulin. It may therefore represent an artifact.

The absolute fasting values of proinsulin in normal subjects ranged between 0.05 and 0.4 ng/ml, representing 5 and 48% of the insulin peak respectively. The fasting

value was unusually high in one patient (P.O.), being greater than that of insulin. After glucose administration, striking changes in the proinsulin concentration were not observed although it tended to rise similarly to insulin. When the values were expressed as percentage of the insulin level, however, the pattern differed. In the first 2 hr after oral glucose, the per cent proinsulin decreased, while towards the end of the test it returned towards fasting levels. These changes were due to the relatively greater increase in insulin concentration after glucose stimulation, rather than being a direct consequence of changing proinsulin levels.

The three obese subjects with abnormal glucose tolerance tests and marked hyperinsulinemia showed higher fasting levels of proinsulin and a greater increase after oral glucose than normal subjects. 1 hr after glucose administration, the absolute values of proinsulin were about three times the fasting levels; and during the hyperglycemic phase they closely paralleled the pattern of insulin secretion. When expressed as percentage of the insulin peak, the values were lower between the first and the third hour because of the higher levels of insulin. Thereafter, the percentage of proinsulin rose due to the rapid fall of insulin. As the high levels of proinsulin coexisted with raised insulin concentrations in these obese subjects, the relative proportions of the two hormones were in the same range observed in the normal group. A typical example is seen in patient S.M. In this subject the values of proinsulin were 2.4 ng/ml 1 hr after oral glucose, but this value represented only 10% of the insulin peak. It thus is clear that the hyperinsulinemia of these obese subjects was not due to high levels of serum proinsulin.

Roth, Gorden, and Pastan (18) observed two components of circulating insulin separated by gel filtration on Sephadex G-50. The larger molecular weight component was called "big" insulin while the second corresponded to pancreatic insulin. After administration of 100 g of glucose to normal subjects, "big" insulin was 0-5% of the plasma insulin concentration at 15 min and 5-28% at 2 hr. Recently Goldsmith, Yalow, and Berson (19) using a similar method assayed 42 samples from 23 patients with an anti-porcine insulin serum which reacted identically on a molar basis with porcine proinsulin and human insulin. The concentration of "big" insulin, measured in normal and obese subjects, and the "big" insulin pattern after oral administration of 100 g of glucose corresponded well with those observed for proinsulin. Moreover, Gorden and Roth (20) have been able to partially convert "big" insulin to "little" insulin by trypsin digestion which is a typical characteristic of proinsulin. For these reasons it appears likely that "big" insulin and serum proinsulin are the same substance. An unexpected finding of our study was the relatively high proportion of proinsulin in fasting samples. The significance of this high basal secretion of proinsulin is not clear at this time.

Although there is evidence that proinsulin functions within the beta cells to ensure the formation of the correct disulfide bonds of insulin in high yields, it is also possible that it has extrapancreatic functions of a regulatory nature, possibly differing in some respects from the familiar effects of insulin. It is probable that proinsulin, after being synthesized on the ribosomes of the beta cells, is transported to the Golgi apparatus converted to insulin and packaged into granules (6, 21). Conversion to insulin may take place within these granules as they mature before secretion. Although there is as yet little data concerning the relative proportion of proinsulin and insulin in the granules, it is possible that the conversion process is not always complete and that a small percentage of the granule protein remains as proinsulin. In this event, liberation of granules after glucose stimulation would result in the secretion of small amounts of proinsulin together with insulin, and the relative proportion of proinsulin would vary depending on the stage of maturation of the granules released. On the other hand, the possibility of secretion of proinsulin or insulin, independent of granule formation, or the preferential release of immature granules or "pre-granules" (22) having a higher content of proinsulin also must be considered. This could be in accord with the hypothesis of the release of newly synthesized hormone from the Golgi region or the selective dissolution of newly formed granules (23, 24).

A number of studies concerning the biological activity of proinsulin have been reported. In the isolated fat cell

method, bovine proinsulin has about 2% the activity of insulin.⁴ On rat epididymal fat tissue and diaphragm muscle, Shaw and Chance (25) found an activity of porcine proinsulin of about 20% when compared on a molar basis with insulin. However, this effect was blocked by Kunitz pancreatic trypsin inhibitor. These authors concluded that porcine proinsulin has no intrinsic biological activity, but that its effect on adipose tissue and diaphragm muscle results from conversion to insulin. At present data concerning the biological activity of human proinsulin are not available. If, like porcine and bovine proinsulin, its activity is quite low, the serum biological activity will be overestimated by the immunoassay procedure. Whether the proinsulin which is secreted into the blood stream shows a biological effect only after its conversion to insulin in peripheral tissues is a question which requires additional study.

The demonstration of the presence of proinsulin in blood focuses attention on the question of the specificity of the immunoassay of plasma or serum insulin. Clearly insulin is not the only circulating protein that is capable of reacting in this type of assay system. The magnitude of error introduced will depend on the concentration of proinsulin and also on the relative reactivity of the antiserum with insulin and proinsulin.

ACKNOWLEDGMENTS

We wish to thank Miss Sooja Cho for excellent technical assistance and Mrs. Helen Watson for aid in preparing this manuscript. We are indebted to Dr. J. Schlichtkrull and Mr. H. Ege of the Novo Company (Copenhagen) for generously providing the human insulin and crude proinsulin for this study.

This research was supported by Grant AM-04931 of the U. S. Public Health Service and by a Grant-in-Aid from Eli Lilly & Company (Indianapolis).

REFERENCES

1. Steiner, D. F., and P. E. Oyer. 1967. The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. *Proc. Nat. Acad. Sci. U. S. A.* **57**: 473.
2. Chance, R. E., R. M. Ellis, and W. W. Bromer. 1968. Porcine proinsulin: characterization and amino acid sequence. *Science (Washington)*. **161**: 165.
3. Steiner, D. F., O. Hallund, A. H. Rubenstein, S. Cho, and C. Bayliss. 1968. Isolation and properties of proinsulin, intermediate forms, and other minor components from crystalline bovine insulin. *Diabetes*. **17**: 725.
4. Frank, B. H., and A. J. Veros. 1968. Physical studies on proinsulin. Association behavior and conformation in solution. *Biochem. Biophys. Res. Commun.* **32**: 155.
5. Rubenstein, A. H., F. Melani, S. Pilakis, and D. F. Steiner. 1969. Proinsulin: secretion, metabolism, immunological and biological properties. *Postgrad. Med. J.* **45**: 475.

⁴ Gliemann, J. 1968. Personal communication.

6. Steiner, D. F., J. L. Clark, C. Nolan, A. H. Rubenstein, E. Margoliash, B. Aten, and P. E. Oyer. 1969. Proinsulin and the biosynthesis of insulin. In *Recent Progress in Hormone Research*. E. B. Astwood, editor. Academic Press, Inc., New York. **25**: 207.
7. Rubenstein, A. H., and D. F. Steiner. 1968. Human proinsulin: some considerations in the development of a specific immunoassay. In *Proceedings of the International Symposium on Early Diabetes*, Marbella, Spain. Academic Press, Inc., New York. In press.
8. Rubenstein, A. H., S. Cho, and D. F. Steiner. 1968. Evidence for proinsulin in human urine and serum. *Lancet*. **1**: 1353.
9. Woods, K. R., and K. T. Wang. 1967. Separation of dansyl-amino acids by polyamide layer chromatography. *Biochim. Biophys. Acta*. **133**: 369.
10. Yalow, R. S., and S. A. Berson. 1964. Immunoassay of plasma insulin. In *Methods of Biochemical Analysis*. D. Glick, editor. Interscience Publishers Inc., New York. **12**: 69.
11. Bailey, J. L. 1957. The preparation of proteins and peptides containing S-sulphonate groups. *Biochem. J.* **67**: 21.
12. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-labeled human growth hormone of high specific activity. *Nature (London)*. **194**: 495.
13. Morgan, C. R., and A. Lazarow. 1963. Immunoassay of insulin: two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats. *Diabetes*. **12**: 115.
14. Davoren, P. R. 1962. The isolation of insulin from a single cat pancreas. *Biochim. Biophys. Acta*. **63**: 150.
15. Wang, S. S., and F. H. Carpenter. 1965. A compositional assay for insulin applied to a search for "Proinsulin." *J. Biol. Chem.* **240**: 1619.
16. Hoffman, W. S. 1937. A rapid photoelectric method for determination of glucose in blood and urine. *J. Biol. Chem.* **120**: 51.
17. Yalow, R. S., and S. A. Berson. 1961. Immunologic aspects of insulin. *Amer. J. Med.* **31**: 882.
18. Roth, J., P. Gorden, and I. Pastan. 1968. "Big insulin": a new component of plasma insulin detected by immunoassay. *Proc. Nat. Acad. Sci. U. S. A.* **61**: 138.
19. Goldsmith, S. J., R. S. Yalow, and S. A. Berson. 1969. Significance of human plasma insulin Sephadex fractions. Program 29th Ann. Meeting of American Diabetes Association. **18**(Suppl. 1): 52. (Abstr.)
20. Gorden, P., and J. Roth. 1969. Circulating insulins. "Big" and "little." *Arch. Intern. Med.* **123**: 237.
21. Lacy, P. E. 1968. Ultrastructural changes of the beta cells during insulin secretion. II. Capri Conference on the "Mechanism and regulation of insulin secretion." *Acta Diabetol. Latina*. **5**(Suppl. I): 436.
22. Logothetopoulos, J. 1966. Electron microscopy of the pancreatic islets of the rat. Effects of prolonged insulin injections. *Diabetes*. **15**: 823.
23. Haist, R. R. 1965. Effects of changes in stimulation on the structure and function of islet cells. In *On the Nature and Treatment of Diabetes*. B. S. Leibel and G. A. Wrenshall, editors. Excerpta Medica Foundation, Publishers, Amsterdam. **12**.
24. Creutzfeldt, W., C. Creutzfeldt, H. Frerichs, E. Perings, and K. Sikinger. 1969. The morphological substrate of the inhibition of insulin secretion by diazoxide. *Horm. Metab. Res.* **1**: 53.
25. Shaw, W. N., and R. E. Chance. 1968. Effect of porcine proinsulin *in vitro* on adipose tissue and diaphragm of the normal rat. *Diabetes*. **17**: 737.