THE BIOSYNTHESIS OF INSULIN AND A PROBABLE PRECURSOR OF INSULIN BY A HUMAN ISLET CELL ADENOMA

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Because of the occurrence of two polypeptide chains in the insulin molecule, it has been proposed by several investigators, most recently by Givol *et al.*,[1] that it may be synthesized *in vivo* as a single large polypeptide chain which is converted by proteolysis to insulin after the native conformation has been established. This mechanism would undoubtedly ensure correct and efficient assembly *in vivo*. However, high yields of insulin have been obtained *in vitro* by recombination of separated or synthetic chains, especially when directing groups are employed.[2] The results of Humbel[3] also are consistent with a separate parallel synthesis of the two chains of angler fish insulin. Furthermore, Wang and Carpenter[4] have looked for a "proinsulin" in pancreatic extracts but have concluded that if such exists, it comprises less than 10 per cent of the total insulin in the pancreas.

The preponderant acinar tissue in the mammalian pancreas and its proteolytic enzymes has restricted most studies of insulin biosynthesis to a few species of teleost fish in which the islet tissue is separate from the pancreas.[5, 6] Insulin-producing tumors of the islets of Langerhans, which occasionally occur in humans, are uniquely suited for the study of insulin biosynthesis since they are composed mainly of β cells and are well separated from the surrounding acinar tissue. We recently obtained part of an islet cell adenoma that was removed from the pancreas of a patient with severe hypoglycemic episodes. When slices from this tumor were incubated with tritiated leucine or phenylalanine, the amino acids were incorporated into insulin. A second labeled protein was separated from the acid-alcohol soluble fraction which could be shown to be related immunologically and chemically to insulin. Treatment of the second fraction with trypsin resulted in its conversion to a form that could not be distinguished from insulin. The results suggest that there is a precursor in the synthesis of insulin and that the precursor is a larger protein than insulin.

Materials and Methods.—D,L-phenylalanine-H^3 (675 mc/mmole) and L-leucine 4,5-H^3 (5.0 c/mmole) were obtained from the New England Nuclear Corporation. Crystalline trypsin (Trypure) was obtained from Novo Terapeutisk, Copenhagen.

Incubation procedure: Approximately 200 mg of slices were prepared from the fresh tumor and placed in two small beakers, each of which contained 5.0 ml of cold incubation medium. The medium was Hank's salt solution[7] containing 320 mg/100 ml of glucose and 20 mg/liter of each of the 18 naturally occurring amino acids. In one beaker approximately 1.5 mc of tritiated phenylalanine was substituted for phenylalanine, while in the other beaker 0.3 mc of tritiated leucine was substituted for leucine. The beakers were incubated 4.5 hr at 37°C in a Dubnoff shaking metabolic incubator under an atmosphere of 95% O_2/5% CO_2.

Extraction procedure: The contents of each beaker were extracted with cold acid-alcohol solution and the extracts partially purified by the method of Davoren.[8]

Gel filtration procedures: Gel filtration was done on 1 × 50-cm columns of Sephadex G-50 (medium grade). These were calibrated with ribonuclease, lysozyme, several trypsin inhibitors, porcine insulin, desoctapeptide insulin, S-sulfo B chain, and glucagon.[9] Insulin appeared to be fully dissociated to monomers in 1 *M* acetic acid solution.[10] Protein was detected by means of ultraviolet absorption at 275 mμ in a Gilford spectrophotometer. Aliquots of fractions from gel

filtration were added to 15 ml of Bray's solution[12] in plastic counting vials and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

Sulfitolysis: Oxidative sulfitolysis was carried out in 8.0 *M* urea by the procedure of Dixon and Wardlaw.[11] The products were resolved by high-voltage electrophoresis on Whatman 3 MM paper using 8 *M* urea, 10% acetic acid, 1% pyridine buffer, pH 4.1, and were visualized by spraying with p-diazosulfanilic acid. Radioactivity was measured in a Vanguard strip scanner with a 4-π windowless detector.

Immunologic procedures: The insulin antisera used for these experiments were prepared in guinea pigs by standard techniques and were capable of binding approximately 80 μg/ml of porcine insulin as determined by immunoassay. Labeled tumor components which were to be tested for immunoreactivity were incubated at 37° for 1 hr, and then for 18 hr at 2°, in the following mixture: 25 μl 0.05 *M* tris-HCl buffer, pH 7.5, 100 μl normal serum *or* antiserum, 5 μl of labeled material (0.5–2.0 μg protein). After addition of 60 μl of 5% sucrose in 0.15 *M* saline, the mixtures were layered on gradients of 5–20% sucrose in 0.15 *M* saline (4.5 ml). Centrifugation was carried out for 20 or 25 hr at 35,000 rpm in the SW-39 rotor at 3°. After centrifugation the tubes were pierced and fractions were collected, including the pellet from the bottom of the tube. These fractions were diluted with 1.0 ml of 0.15 *M* saline, and assayed for optical density at 280 mμ and then for radioactivity.

Amino-terminal analysis: This procedure was an adaptation for small samples of the dinitrophenylation method of Sanger.[13] The DNP-proteins were hydrolyzed in 6 *N* HCl for 40 hr at 105°. Aliquots of ether extracts of the hydrolysates were assayed for radioactivity.

Results.—When the partially purified acid-alcohol extract of the tumor slices incubated with leucine-H^3 was subjected to gel filtration on Sephadex G-50, a pattern of optical density and radioactivity appeared as shown in Figure 1. The three peaks of leucine-labeled radioactive material were arbitrarily designated *a*, *b*, and *c*. Peak *c* was symmetrical both with respect to optical density and radioactivity, and its elution position corresponded closely to that of authentic porcine insulin, which differs from human insulin by a replacement of alanine with threonine at position 30 of the B chain. The amount of protein in this peak exceeded 1.0 mg and appeared to be mainly insulin by immunological assay with the Berson and Yalow technique.[14] (Assays were kindly performed by Mrs. Lydia Kursteins of the Department of Medicine.) Hence, it can be estimated that the tumor contained as much as 10 mg of insulin/gm tissue. The specific activity of the insulin amounted to about 20 μc/mg. The more rapidly eluted peak *b* of radioactive material was not associated with any appreciable amount of ultraviolet absorbing material, while peak *a* appeared to be a mixture of ultraviolet absorbing materials appearing in the void volume of the column (see Fig. 1). The elution diagram of the phenylalanine-labeled protein was essentially the same as that for the leucine-labeled protein. The fractions containing the center regions of peaks *a*, *b*, and *c*,

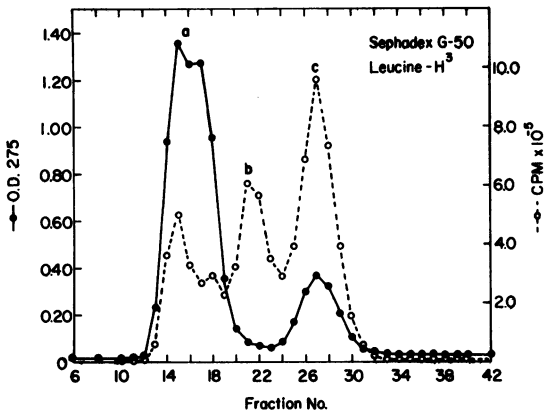

FIG. 1.—Elution pattern of H^3-leucine-labeled acid-alcohol soluble protein extracted from the adenoma incubation mixture. The void volume of this column extended to fraction 15.

respectively, from each experiment were combined, evaporated to dryness, dissolved in 1.0 ml of 0.01 *N* HCl, and stored at −18°.

Identification of peak c as insulin: The following observations identified the material in peak *c* as insulin: (1) Its elution volume on Sephadex G-50 was essentially identical with that for porcine and bovine insulin. (2) It was bound by insulin antisera and displaced from antibody by porcine insulin (see Fig. 2). (3) It migrated with porcine insulin on electrophoresis in 8 *M* urea, 10 per cent acetic acid, 1 per cent pyridine at pH 4.1.[11] (4) Sulfitolysis of leucine-labeled component *c* followed by electrophoresis yielded labeled A and B chains in a ratio of 2 to 4, the molar ratio of leucine in the A and B chains, respectively, of human insulin. Under the same conditions the phenylalanine-labeled component *c* yielded only a labeled B spot in accord with the absence of phenylalanine from the A chain. (5) Phenylalanine-labeled component *c* released labeled heptapeptide when digested with trypsin (see Fig. 3). Leucine-labeled component *c* did not yield labeled heptapeptide under the same conditions. The composition of the heptapeptide (B-22 to 29) is gly.*phe*.*phe*.tyr.thr.pro.lys.[15]

The above findings are all in accord with the known structure of human insulin.[16]

The relationship of component b to insulin: The fact that peak *b* had a very high specific activity prompted a further examination of this material. An immunological relationship between component *b* and insulin was demonstrated as follows: labeled material from peak *c* or *b* was incubated with an excess of guinea pig anti-insulin serum or normal serum, and then subjected to zone centrifugation on sucrose gradients. By this means the antigen-antibody complex was separated completely

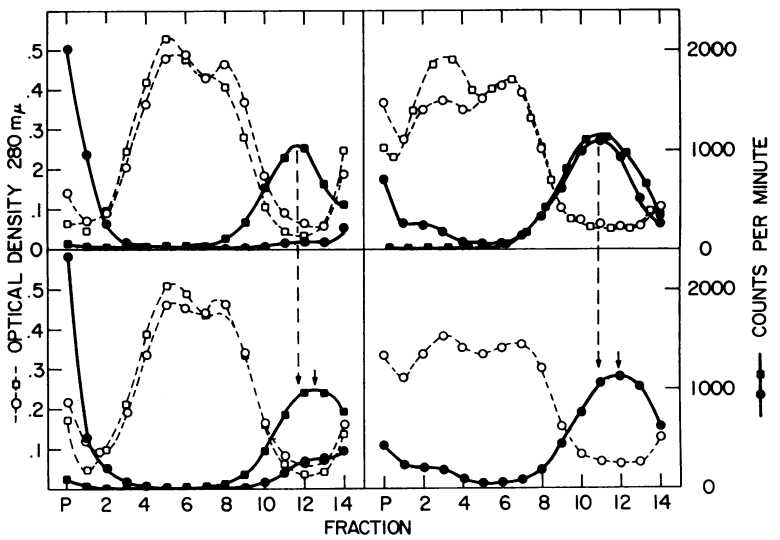

FIG. 2.—Distribution of labeled protein on sucrose gradients after incubation of tumor components *b* or *c* with nonimmune guinea pig serum (*squares*), or anti-insulin serum (*circles*). The upper panels show the results obtained with leucine-labeled component *b*, while the lower panels represent the results obtained with leucine-labeled component *c* (insulin). In the experiments shown in the two right panels, 75 μg of nonlabeled porcine insulin was included in the incubation mixtures. These were centrifuged 25 hr, whereas those shown on the left were centrifuged 20 hr, at 35,000 rpm. Dotted arrows indicate faster sedimentation of free component *b* as compared with free component *c*.

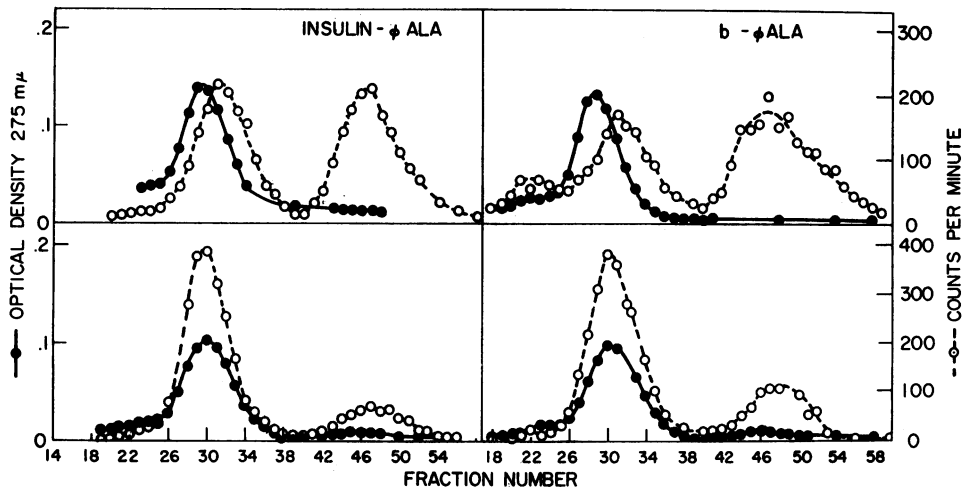

FIG. 3.—Conversion of phenylalanine-labeled component *b* and component *c* (insulin) to desoctapeptide form by trypsin. The reaction mixture contained 90 μl 0.05 *M* tris HCl (pH 7.5), 15 μl of component *b* or *c* in 0.01 *N* HCl, and 5 μl trypsin solution (2 mg/ml). Incubation was carried out for 2 hr at 37°. In the experiments shown in the upper panels, 1 mg of porcine insulin was added *after* incubation. In the experiment shown below, 1 mg of porcine insulin was included in each reaction mixture during incubation. Gel filtration was carried out as described in *Methods*. The second radioactive peak (fractions 44–50) corresponds in its position to that expected for heptapeptide.[15]

from unreacted material which remained in the upper half of the tubes. As shown in Figure 2, both the phenylalanine- and leucine-labeled insulins (*c*) became bound to globulin complexes when they were incubated with guinea pig anti-insulin serum. Addition of 75 μg of nonlabeled porcine insulin before incubation displaced the labeled material from the globulin complexes, as would be anticipated for a specific antigen-antibody reaction. Labeled component *b* behaved essentially identically in this system to component *c*, and it also was displaced by porcine insulin from the antibody. (It was noted, however, that component *b* sedimented more rapidly on the sucrose gradients than did component *c* (Fig. 2), in keeping with its larger molecular size, i.e., mol wt ~10,800, as estimated by gel filtration.[9]) Since the separated chains of insulin are not reactive with anti-insulin sera,[17] it was concluded that component *b* must be closely related immunologically to native insulin.

In view of the limited cleavage of insulin brought about by trypsin, it was decided to incubate component *b* with this protease. The results of an experiment in which limiting amounts of trypsin were used are shown in Figure 4. As little as 0.1 μg of trypsin (1 μg/ml) in 30 min almost completely converted leucine-labeled component *b* to a form which eluted with porcine insulin. After incubation with higher concentrations of trypsin (100 μg/ml), the radioactivity shifted to a position corresponding to that expected for desoctapeptide insulin (Fig. 5). Also, with the larger amount of trypsin a shoulder began to appear on the right-hand side of the main peak of radioactivity when labeled component *b* was digested, but not when labeled insulin was digested (Fig. 5). Acetyl trypsin also was effective in this conversion, but chymotrypsin degraded both component *b* and insulin much more extensively. Insulin was not released from the protein by extremes of pH, high ionic strength, 50 per cent acetic acid, or 8 *M* urea.

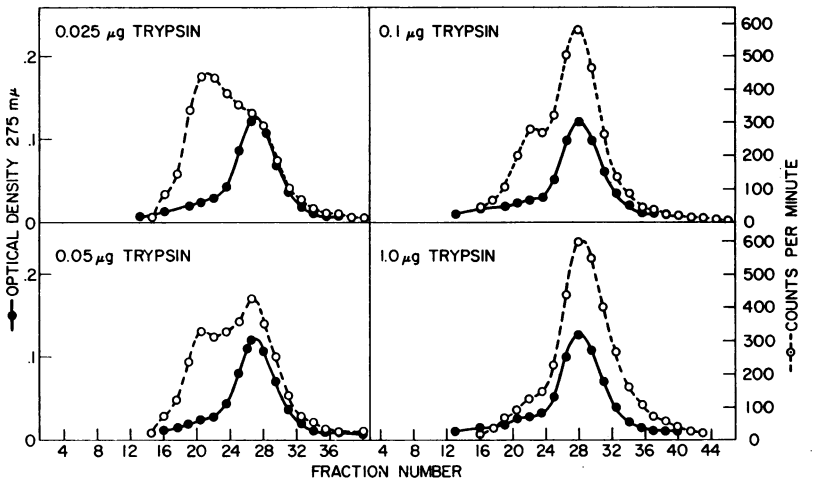

FIG. 4.—Conversion of leucine-labeled component *b* during incubation with varying amounts of trypsin. The reaction mixture contained 90 μl 0.05 *M* tris HCl (pH 7.5), 15 μl component *b* in 0.01 *N* HCl, and 5 μl trypsin solution containing the amounts of trypsin shown. After incubation at 37° for 30 min, 0.9 ml of 1 *M* acetic acid, 5 μl of 1.0 *N* HCl, and 1 mg porcine insulin were added to each. Gel filtration on Sephadex G-50 was carried out as described in *Methods*.

It was important to compare the effect of tryptic digestion on phenylalanine-labeled insulin and component *b*, since the tryptic heptapeptide from insulin contains two of the three phenylalanine residues of the B chain. In the experiments shown in the upper panels of Figure 3, phenylalanine-labeled insulin (*c*) or component *b* was incubated with enough trypsin to produce appreciable amounts of desoctapeptide insulin.[15] Porcine insulin was added *after* incubation to serve as a marker. Both components *b* and *c* gave rise to desoctapeptide insulin which eluted somewhat more slowly than unaltered insulin (OD peak), and both gave rise to labeled peptide material (second radioactive peak) in about equal amounts. This result represents about 80 per cent conversion to the desoctapeptide form.

In the experiment shown in the lower panel of Figure 3, 1 mg of unlabeled porcine insulin was included in each reaction mixture *during* the trypsin digestion. Now the peaks of optical density from porcine insulin and of radioactivity from labeled components *c* or *b* were congruous, and both components gave rise to lesser amounts of labeled heptapeptide.

The preceding experiments showed that insulin can be released by tryptic digestion of the *b* component, but did not help to decide whether this heavier protein is a complex of insulin with another protein, or is a discrete protein, which contains insulin's sequences bound covalently to other peptide material. Accordingly, sulfitolysis of component *b* in 8 *M* urea[11] was carried out to determine whether the chains of insulin would be released. After sulfitolysis, leucine-labeled component *b* did not give rise to either a labeled A or B chain, as did leucine-labeled insulin. The radioactivity on electrophoretograms occupied an intermediate position between the A and B chains. When sulfitolysis was carried out after treatment with trypsin, leucine-labeled component *b* yielded labeled material corresponding to the A and B chains of insulin on electrophoresis. When cal-

culated relative to component *c*, component *b* gave rise to about 65–70 per cent as much A chain on the basis of total initial radioactivity. (The S-sulfo A chain of insulin was used for this estimate because on electrophoresis it separates most cleanly from all other components.)

Gel filtration was utilized also to assess the effect of sulfitolysis upon component *b*. Leucine-labeled component *b* was mixed with porcine insulin. The mixture was subjected to sulfitolysis, and then diluted with 8 *M* urea, 0.1 *N* NH_4OH, and sufficient EDTA to bind the copper ions from the sulfitolysis mixture. The products were separated by gel filtration on Sephadex G-50 in 8 *M* urea, 0.1 *N* NH_4OH. The exclusion limits of the gel were lowered by this solvent. Porcine insulin was cleaved to S-sulfo A and B chains which eluted together, as shown by the solid line in both upper and lower panels of Figure 6. Sulfitolysis of component *b* did not yield appreciable amounts of radioactivity in the region of the chains. The bulk of the radioactivity still eluted in a position earlier even than that of intact insulin, as indicated by the arrow in the upper panel of Figure 6. Sulfitolysis of component *c* yielded a peak of radioactivity corresponding closely to the peak containing the S-sulfo chains of porcine insulin (Fig. 6, lower panel), as did sulfitolysis of component *b* after trypsin treatment.

End group analysis: It was possible to determine if the N-terminus of component

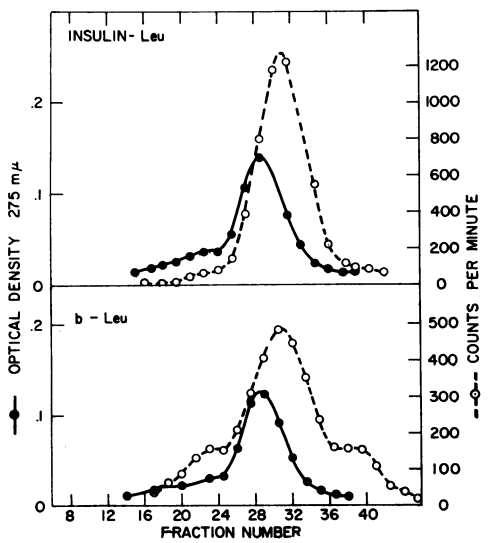

FIG. 5.—Conversion of leucine-labeled components *b* and *c* (insulin) to desoctapeptide form by trypsin. Conditions were similar to those used for the experiment shown in Fig. 4, except that more trypsin (10 μg) was added to each reaction mixture. Porcine insulin (1 mg) was added *after* incubation.

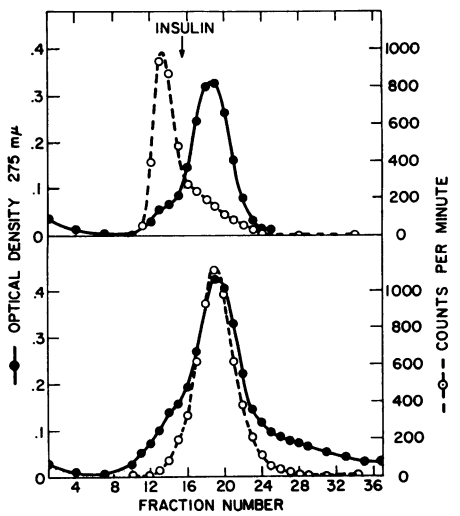

FIG. 6.—Gel filtration on Sephadex G-50 in 8 *M* urea, 0.1 *N* NH_4OH, of leucine-labeled component *b* or *c* after sulfitolysis in the presence of 1.5 mg porcine insulin. In the experiment shown in the upper panel, 25 μl of component *b* (dried *in vacuo*) were incubated 20 min in 100 μl of the sulfitolysis mixture of Dixon and Wardlaw.[11] In the experiment shown in the lower panel, 10 μl (dried *in vacuo*) of component *c* (insulin) were incubated under the same conditions. Arrow indicates elution position of intact porcine insulin in this system. The void volume of the column extended to fraction 11. (See text for experimental details.)

TABLE 1

RADIOACTIVITY OF DINITROPHENYL DERIVATIVES PREPARED FROM PHENYLALANINE- OR LEUCINE-LABELED INSULIN OR COMPONENT *b*

Sample	Initial total radioactivity (cpm)	Total radioactivity dinitrophenyl derivatives (cpm)	Recovery* of radioactivity % of theory
b-Phe	17,450	2740	47
*b*t-Phe†	17,450	2780	48
c-Phe	20,915	3640	52
b-Leu	30,000	61	<1
*b*t-Leu†	30,000	72	<1

* Based on the known distribution of phenylalanine in the B chain (only one of three occupies the N-terminal position).
† Incubated with trypsin (10 μg/ml) for 30 min at 37° before dinitrophenylation (conditions as described in the legend for Fig. 5).

b was phenylalanine as in the case of the mammalian B chains because phenylalanine had been used to label both insulin and component *b*. In the experiment shown in Table 1, the presence of radioactivity in dinitrophenyl derivatives extracted after acid hydrolysis is compared in a series of samples. From these data it is clear that a labeled N-terminal group is present in the phenylalanine-labeled fractions, but not in the leucine-labeled fractions. Moreover, the comparable recovery of dinitrophenyl radioactivity from phenylalanine-labeled component *b* as compared with insulin (*c*), and the lack of effect of prior trypsin treatment on this result, suggests strongly that the normal B chain N-terminal phenylalanine is free in component *b*.

Discussion.—From the preceding data it is evident that component *b* is a protein containing the amino acid sequences of insulin, and also having the same N-terminal amino acid as does the B chain of insulin. We have no information about the N-terminal amino acid of the A chain, which was not labeled in these experiments. However, the absence of a labeled A chain after sulfitolysis of component *b*, prior to trypsin treatment, makes it unlikely that the A chain is present in its usual form. Moreover, in view of the failure of sulfitolysis in 8 *M* urea to lower its molecular size significantly, as judged by gel filtration, it may be concluded that the molecule consists of one or more polypeptide chains that are larger than insulin. These observations are all consistent with the hypothesis that component *b* is a precursor in the biosynthesis of insulin. It is possible that this precursor protein consists of a single polypeptide chain beginning at its N-terminal end with the B chain sequence of insulin, terminating with the A chain sequence, and bearing an additional stretch of polypeptide between the normal chain sequences. The analysis of Humbel,[3] which rules out this possibility, requires the assumption that each proline in angler fish insulin is inserted by the same tRNA species. If this is not the case, the specific activities of the proline residues along the chains of insulin could reflect different rates of turnover of individual prolyl tRNA fractions, rather than their relative positions along the chains.

We recently have extracted from several commercial preparations of crystalline insulin small amounts (<1%) of a protein which behaves similarly to component *b* on gel filtration, and which can be converted by trypsin digestion to material which corresponds closely in its elution position to that of insulin. It appears, therefore, that small amounts of the precursor protein can be obtained from purified insulin, with which it may crystallize. The amino acid composition of this fraction is similar to, but not identical with, that of insulin.

Summary.—Tritiated phenylalanine and leucine were incorporated into acid-alcohol soluble protein during incubation of slices from a human islet cell adenoma *in vitro*. When partially purified acid-alcohol extracts of the incubation mixtures were subjected to gel filtration on columns of Sephadex G-50, two peaks of labeled material were separated, both of which reacted with guinea pig anti-insulin serum. The more slowly eluted peak was apparently identical with normal human insulin and was more abundant than the earlier eluted component. A large proportion of the early eluting material could be converted to a form indistinguishable from authentic insulin by digestion with small amounts of trypsin. N-terminal amino acid analysis of phenylalanine-labeled insulin and the early component revealed the presence in both of a free N-terminal phenylalanine residue. The evidence available indicates that the immunoreactive material consists of a larger protein molecule of mol wt about 10,800 which includes the amino acid sequences of insulin in a form which can be released by limited proteolysis.

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