choline was required for the conversion of pneumococci to the competent physiological state, in which these bacteria can absorb DNA molecules and undergo genetic transformation (*14*).

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Insulin Biosynthesis: Evidence for a Precursor

Abstract. *Human islet cell tumor tissue and isolated islets of Langerhans from rats incorporated radioactive amino acids in vitro into insulin and a larger acid-alcohol soluble protein which could be separated from insulin by gel filtration. The amino acids were incorporated into the larger protein earlier than into insulin; only after incubation of islets for approximately 30 minutes did radioactivity begin to appear in insulin. The transfer of about 70 percent of the radioactivity of the larger protein to insulin was demonstrated in the absence of new peptide bond synthesis (cycloheximide), or during incubation with unlabeled amino acid (chase). The results indicate that the larger protein is a precursor in the biosynthesis of insulin. The name "proinsulin" is suggested for this protein.*

Slices from a human, insulin-producing β-cell tumor incorporated labeled amino acids into insulin and another acid-alcohol extractable protein (designated component *b*) which is larger than insulin (*1*). The latter is related immunologically to insulin, and insulin apparently is released from it upon incubation with small amounts of trypsin. It was postulated that the larger protein is a precursor of insulin which had accumulated in small amounts in the tumor. The studies to be presented here on the time course of insulin biosynthesis were carried out with another human β-cell tumor, and also with isolated islets of Langerhans from rat pancreas. The results support the earlier interpretation.

When material from a second insulin-producing tumor became available, a study was undertaken to examine the time course of incorporation of radioactive amino acids into component *b* and insulin (*2*). It was assumed that if component *b* is a precursor of insulin, radioactivity would appear first in it, and that upon subsequent incubation radioactivity would be transferred into the fraction containing insulin.

Slices from the tumor (10 to 20 mg) were incubated at 37°C for various intervals in small plastic tubes with the medium containing glucose (3 mg/ml) and L-leucine-H^3 (5 c/mmole) or L-phenylalanine-H^3 (3 c/mmole) described previously (*1*). In order to terminate labeling and initiate a chase, unlabeled leucine or phenylalanine was added to the medium at various times during incubation to give a final concentration of 30 μg/ml. After incubation each mixture was frozen at −79°C and transported on dry ice from Iowa City to Chicago, where the study was completed.

Data on the incorporation of amino acids into the acid-alcohol insoluble protein of the slices (Fig. 1) indicated that protein synthesis proceeded at a nearly linear rate. When unlabeled amino acid was added to dilute the labeled amino acid about 100 fold, the rate of incorporation of radioactivity decreased markedly. The acid-alcohol soluble fraction was separated and partially purified, and then it was passed through columns of Sephadex G-50 equilibrated with 1*M* acetic acid as described previously (*1*). The results are shown in Fig. 2. After incubation for 40 minutes label was present in a peak which eluted at a position corresponding to that of component *b* derived from the original tumor, but no label eluted in a position corresponding to that of added authentic porcine insulin. After incubation for 80 minutes peak *b* was higher and a small shoulder of radioactivity had appeared in the region of the insulin marker which, when separated and rerun (Fig. 2, inset), indicated the presence of a small amount of labeled insulin. Essentially similar results were obtained in experiments where a chase was carried out over the period from 40 to 80 minutes. After incubation of slices for 210 minutes a definite peak of radioactivity eluted in a position corresponding to that for insulin. The tubes containing peaks *b* or *c* were combined, the acetic acid was evaporated, and the protein was dissolved in 0.01*N* HCl for further analysis.

Material from both peaks *b* and *c* was bound by antibody to porcine insulin as noted previously (*1*). At least 50 percent of the radioactivity from peak *b* from this tumor was converted

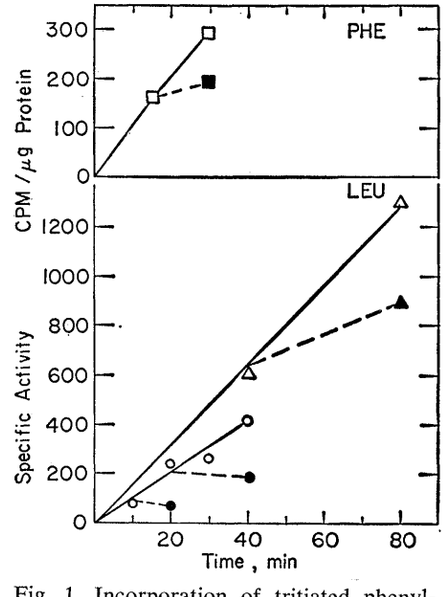

Fig. 1. Incorporation of tritiated phenylalanine (Phe) or leucine (Leu) into the acid-insoluble protein fraction of islet tumor slices. The residues obtained after acid-alcohol extraction and centrifugation were heated to 100°C in 5 percent trichloroacetic acid, then washed three times with 5 percent trichloroacetic acid and three times with 95 percent alcohol. The precipitates were dissolved in 0.3*N* KOH, their radioactivity was measured in Bray's solution (*15*) in a liquid-scintillation counter, and the protein concentration was measured by the method of Lowry (*16*). Solid symbols indicate samples in which excess unlabeled amino acid was added at the time indicated by the dashed line.

by treatment with small amounts of trypsin to a form which eluted with insulin from Sephadex G-50. Both leucine-labeled peak *c* and trypsin-treated peak *b* gave rise to radioactive peaks corresponding in their positions to the chains of porcine insulin after sulfitolysis and paper electrophoresis in 8*M* urea at *p*H 4.1 (*1*) or gel filtration on Sephadex G-75 in 50 percent acetic acid (*3*). When phenylalanine-labeled material from peak *b* or *c* was digested more extensively with trypsin, a labeled peptide was released which behaved identically on electrophoresis (1 percent ammonium carbonate, *p*H 8.9) and paper chromatography (1 percent acetic acid: *sec*-butanol, 1 : 1) to the heptapeptide containing phenylalanine that is known to be released from insulin by trypsin digestion (*4*). No label was found in this peptide when the material labeled with leucine was digested with trypsin. It is clear, therefore, that most of the radioactive protein in peak *c* is insulin, while that in peak *b* corresponds in large part to the previously identified larger protein. It is of interest that, unlike the first tumor, which contained a large amount of insulin, presumably stored in secretory granules, this tumor contained such a small amount that it was not detectable by measurement of optical density (Fig. 2, lower panel). This finding is in agreement with the histologic examination which disclosed that the first tumor contained abundant granules while the second one did not. Both tumors were able to make insulin, however, as indicated also by the clinical findings of severe recurrent episodes of hypoglycemia.

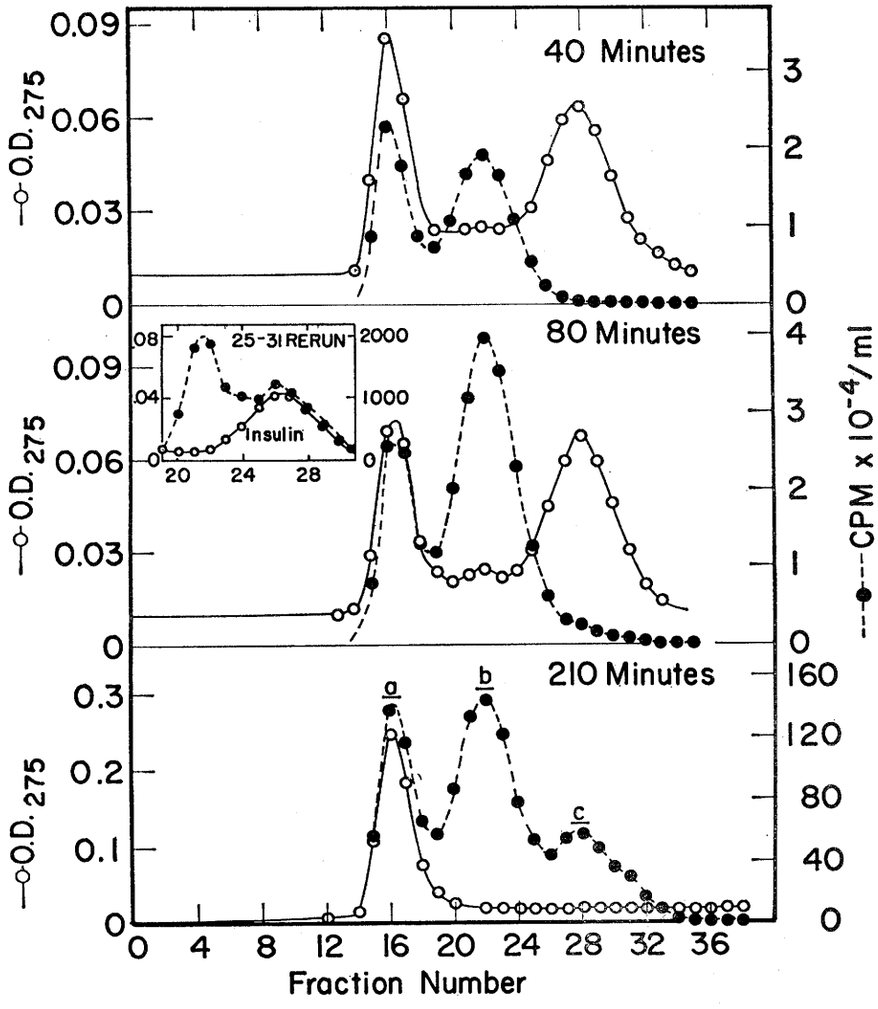

Fig. 2. Gel filtration elution diagram of partially purified acid-alcohol soluble protein extracted from incubated islet tumor slices. The slices were incubated as described in the text with medium containing L-leucine-4,5-H^3 for the intervals shown on each panel. For the two experiments shown in the upper panels about 20 mg of slices were incubated in 0.5 ml of medium, while for the experiment in the lower panel, about 200 mg of slices were incubated in 5.0 ml of medium. One milligram of authentic porcine insulin was added to the 40- and 80-minute incubation mixtures just before acid-alcohol extraction. No carrier insulin was added to the larger 210-minute incubation mixture before extraction. The absence of a significant O.D. peak near tube 28, the elution position of insulin, indicates that this 200-mg sample of tumor contained less than 10 to 20 μg of insulin. The column was 1 by 50 cm, packed with Sephadex G-50 (medium), equilibrated, and eluted with 1*M* acetic acid. The void volume extended to fraction 16.

Because of the infrequent occurrence of β-cell tumors in man and other difficulties associated with obtaining this material in a suitably fresh form, we turned our attention to the possibility of utilizing a laboratory animal instead. Whole pancreas from either adult or fetal rats proved unsuitable because of the heavy labeling of zymogens and the presence of large amounts of proteolytic enzymes. We therefore have used the procedure developed by Moskalewski (*5*) as modified by Lacy and Kostianovsky (*6*) to isolate intact islets of Langerhans from rat pancreas. This method employs collagenase (Worthington, purified) to digest the supporting collagenous framework of the organ, and when carried out under controlled conditions, large numbers of islets free of acinar cells are liberated. The islets can be picked up easily with Lang-Levy micropipettes with the aid of a dissecting microscope.

Isolated islets were washed several times with Hanks medium containing 200 μg of bovine serum albumin (BSA) per milliliter, and 10 to 15 of them were transferred to 50 μl of incubation medium on a plastic surface (Falcon culture dishes) or in the tip of a tapered 12-ml glass centrifuge tube. The incubation medium contained 100 μc of L-leucine-H^3 per milliliter (5 c/mmole) and was similar to that used previously except for the addition of 200 μg of BSA per milliliter and 1 μg of pancreatic trypsin inhibitor per milliliter. The addition of the albumin was necessary to prevent adsorption of insulin and other labeled proteins to glass surfaces. Incubation was carried out at 37°C under an atmosphere of 95 percent O_2, 5 percent CO_2. The islets could be incubated in this manner for periods up to 6 hours without visible change, and during this time protein synthesis proceeded at an essentially linear rate. After incubation, 1 mg of bovine insulin was added as carrier, and the islets and medium were homogenized in small tapered glass homogenizers with

acid-alcohol containing unlabeled leucine.

After partial purification (*1*), the acid-alcohol soluble protein was fractionated by gel filtration on 1- by 50-cm columns of Sephadex G-50. In order to recover the labeled protein quantitatively it was necessary to coat the glass tubes used to collect fractions with bovine serum albumin (10 mg/ml in 50 percent acetic acid). After gel filtration the carrier insulin peak was located by optical density at 275 mμ and the radioactivity of the fractions was measured in a liquid scintillation counter. Under these conditions about 8000 count/min were incorporated into the total acid-alcohol soluble fraction per hour when the islets were obtained from rats given either cortisone acetate (5 mg/day) or 10 percent sucrose in the drinking water. Islets from fasted rats were not studied.

Figure 3 shows the elution pattern of the leucine-labeled, acid-alcohol soluble protein from incubated rat islets. In contrast to the complex pattern obtained with whole pancreas, only two major peaks of radioactivity were seen. With short incubation periods of up to 1 hour most of the radioactivity appeared as a well-defined peak which corresponded in its elution position to that for the labeled component *b* found in both human tumor preparations. By 60 minutes a shoulder of radioactivity was present at a position corresponding closely to that for bovine insulin, and with time this peak increased relative to the *b* peak. Radioactive protein from peak *c* yielded material corresponding to the A and B chains of insulin after sulfitolysis and electrophoresis at *p*H 4.1 in 8.0*M* urea or in 30-percent formic acid. The labeled chains were also separated and identified by means of gel filtration on Sephadex G-75 in 50 percent acetic acid. However, unlike labeled human insulin, two radioactive spots were found in the region of the B chain derived from the carrier bovine insulin. One of the peaks moved slightly more rapidly toward the cathode than did the B chain of bovine insulin. This mobility would be expected for the B chain of rat insulin I which contains an additional lysine at position B-3 (*7*). The second peak moved slightly less rapidly toward the cathode than the bovine B chain, un-

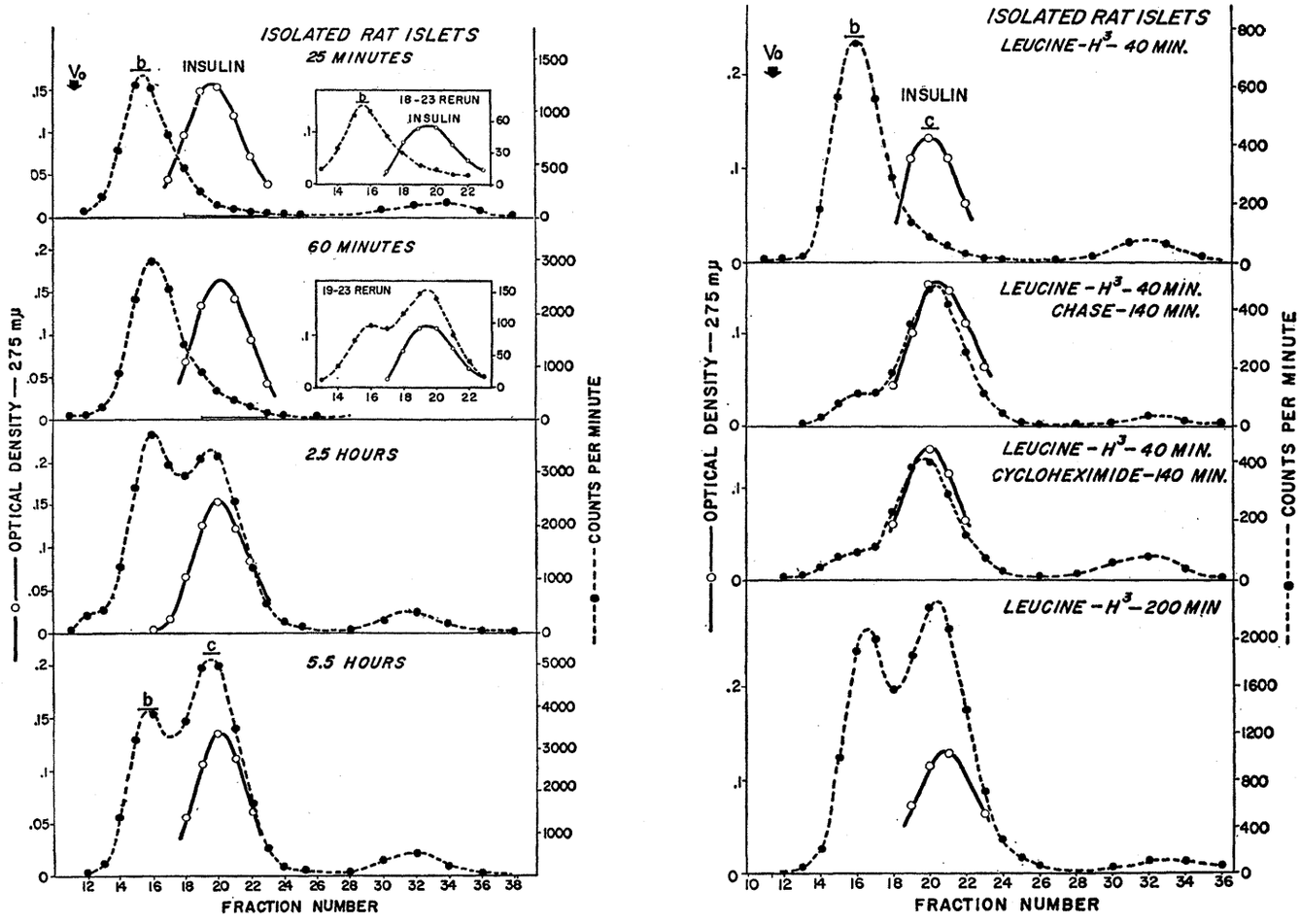

Fig. 3 (left). Elution pattern from Sephadex G-50 of leucine-H³ labeled acid-alcohol soluble protein extracted from islets of Langerhans isolated from rats. The islets were incubated in vitro as described in the text for the interval shown on each panel. Optical density measures bovine insulin, which was added as a carrier before extraction of the islets. The elution position of peak *b* is identical with that of labeled component *b* from the human tumors. Peak *c* contains mainly labeled rat insulin. The contents of fractions 18 to 23 from the 25- and 60-minute incubation mixtures were combined, dried *in vacuo*, and refractionated on the Sephadex column, with the results as shown in the insets. Fig. 4 (right). Evidence for the transfer of radioactivity from peak *b* to peak *c* (insulin) during incubation of isolated islets of Langerhans. Upper panel shows elution pattern of labeled acid-alcohol soluble protein extracted after incubation for 40 minutes. The results in the two center panels demonstrate the transfer of radioactivity from peak *b* to *c* during subsequent incubation for 140 minutes in the presence of cycloheximide (250 μg/ml) or of 100-fold excess of unlabeled L-leucine. The lower diagram shows the pattern of radioactivity found after 200 minutes of incubation without intervention. Optical density measures added bovine insulin. See text for experimental details.

like the B chain of rat insulin II (*7*) which should have the same mobility as the bovine B chain.

The following order was found with respect to incorporation of tritium-labeled amino acids of comparable specific activity into peaks *b* and *c* of the acid-alcohol soluble fraction: leucine ~12; phenylalanine ~6; methionine ~1; tryptophan, 0. The composition of rat insulin with respect to these amino acids is 6 : 3 : ½ : 0 residues/molecule respectively (*7*).

Incubation of leucine-labeled material from peak *b* with TPCK-treated trypsin (*8*, *9*) resulted in its conversion to a form which eluted with bovine insulin on gel filtration, and which also yielded labeled A and B chains upon sulfitolysis. These results indicate that the radioactive protein in peak *b* is similar to the labeled protein fraction from the human tumors designated component *b*. When an appropriate pulse-chase experiment was carried out with intact rat islets, the data shown in Fig. 4 were obtained. After incubation for 40 minutes at 37°C label was found mainly in the *b* region. At this time the incubation medium was withdrawn and replaced with fresh medium containing unlabeled leucine at a concentration of 300 μg/ml. Incubation was continued for an additional 140 minutes, during which interval about 70 percent of the total radioactivity present in peak *b* at 40 minutes moved to a position corresponding to that for bovine insulin.

In the same experiment cycloheximide was added to the medium in some tubes at a concentration previously determined to be sufficient to inhibit protein synthesis of whole pancreas in vitro by 95 percent. It was added after incubation for 40 minutes, and incubation was then continued for an additional 140 minutes. As shown in Fig. 4, about 70 percent of the radioactivity again moved from the *b* region to a position corresponding to that of peak *c* during the period of inhibited protein synthesis.

The preceding experiments demonstrate that the *b* component is present in insulin-synthesizing tissues derived from rats and man. Moreover, this component becomes labeled earlier than does insulin in both tissues, and its radioactivity is transferred to insulin as incubation proceeds in the absence of new or labeled peptide bond synthesis. Our earlier studies indicate that component *b* is a larger protein than insulin and that it contains the amino acid sequences of insulin in a form which can be released by limited tryptic proteolysis (*1*). The fact that component *b* reacts with antibodies to insulin suggests that some antigenic attributes of the native insulin molecule are already present in its structure. The labeling data reported here support our earlier interpretation that component *b* is a precursor in the biosynthesis of insulin. It might be less cumbersome, therefore, to designate this material "proinsulin."

While the existence of proinsulin has often been predicted, it has not been demonstrated previously. However, ultrastructural and fractionation studies have indicated many similarities in the synthetic and secretory mechanisms of the pancreatic acinar and the β cells (*10*, *11*). It is of particular relevance that in the exocrine cells approximately 40 to 60 minutes elapse before newly synthesized zymogen protein first begins to appear in zymogen granules (*10*).

The results of Bauer *et al.* (see *12*) indicate that there may be a similar delay in the transfer of newly synthesized insulin into the granule fraction in the β cells. Moreover, Howell and Taylor (*13*) have found a delay of about 1 hour before newly synthesized insulin is secreted into the medium from slices of rabbit pancreas incubated in vitro.

Our finding of a delay of at least 30 minutes before significant amounts of labeled insulin appear during incubation of islets in vitro suggests that during the intracellular transport of proinsulin, from the region in the rough endoplasmic reticulum where it presumably is formed to the secretory granules, a proteolytic process takes place which results in the liberation of insulin from proinsulin. While it would seem to follow from this kind of biosynthetic mechanism that the precursor form of insulin may exist to protect insulin or aid in its transport within the cell, it is perhaps more likely that it may aid in the correct assembly of the native structure of the molecule. On the other hand, proinsulin may not serve any purpose, but could represent a vestige of some evolutionary ancestor of the insulin molecule.

It usually has been assumed that either insulin or its free A and B chains are the first products of insulin biosynthesis. However, the chains of insulin are not reactive with antiserums to insulin nor are they biologically active until combined. The existence of a precursor which has immunological properties similar to insulin, and which may possess reduced or novel biological activity, should permit the recognition of disorders due to the loss of the enzymatic mechanism(s) necessary for the conversion of the precursor to the normal hormone.

It is now generally recognized that in many adult-onset diabetics, and in the early stages of juvenile diabetes, plasma insulin levels appear to be normal or elevated as measured immunologically. This finding could be explained by the presence in the circulation of an abnormal form of insulin having immunological attributes of insulin but having reduced or altered biological activity. Elliott *et al.* (*14*) have presented evidence that an abnormal form of insulin is present in the serum of juvenile diabetics. Its activity is not destroyed as rapidly by an insulin-degrading enzyme system in vitro as is the insulin of normal individuals. The structure of this abnormal insulin is not known.

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