Abstract.—This communication reports the biosynthesis of insulin in the bovine fetal pancreatic slices in vitro. Double-chain proinsulin and insulin were found as major components in the mitochondrial-granule fraction of bovine fetal pancreas. Tritiated leucine was incorporated into a single-chain proinsulin, a double-chain proinsulin, and insulin. Subcellular fractionation of the slices incubated with tritiated leucine showed that radioactive single-chain proinsulin was present in the deoxycholate-soluble microsomal fraction, deoxycholate-insoluble microsomal fraction, and mitochondrial-granule fraction. Labeled double-chain proinsulin and insulin were present in the deoxycholate-soluble microsomal and mitochondrial-granule fractions. These results are consistent with the hypothesis that insulin is synthesized as a single-chain polypeptide on the ribosomes, and that intracellular proteolysis in the subcellular membranous organelles and beta-granules converts the single-chain proinsulin to insulin via a double-chain intermediate.

Introduction.—A precursor protein in the biosynthesis of insulin has been demonstrated in human islet adenoma tissue, in isolated rat islets of Langerhans,1-2 and in slices of bovine fetal pancreas.4 This precursor protein is a single-chain polypeptide larger than insulin, is immunologically reactive with anti-insulin antibody, and is convertible to desalanyl insulin by limited trypic hydrolysis. Bovine proinsulin has been obtained from crystalline insulin preparations in several laboratories,5-8 and the amino acid sequence of porcine proinsulin is now known.9 A double-chain proinsulin has also been obtained from crystalline bovine insulin.5,7 It is possible that both proteins are involved in the biosynthesis of insulin, the single-chain being the precursor and the double-chain an intermediate. Experiments reported here were undertaken to study (1) the incorporation of radioactive leucine by slices of bovine fetal pancreas into these two proteins and into insulin and (2) the distribution of these proteins in subcellular fractions.

Materials and Methods.—Incubations of tissue slices and preparation of subcellular fractions: Fresh bovine fetal pancreatic slices (3 gm) were incubated at 37° under 95% O2-5% CO2 in 10 ml of Hank’s buffer,10 containing 320 mg % of glucose, 20 mg/liter of each of the 18 naturally occurring amino acids, 1 mc of leucine-4,5-H2 (specific activity, 5.9 mc/mmole, New England Nuclear Corp.), and Trasylol (protease inhibitor, 1 X 104 units, FBA Pharmaceuticals). After incubation, the slices were rinsed several times with ice-cold 0.25 M sucrose and homogenized in 15 ml of 0.25 M sucrose at 4° in a glass Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was then
centrifuged to give the following fractions: (I) nuclei, zymogens, and cell debris, at \(8.41 \times 10^4\) g/min; (II) mitochondrial-granule, at \(2.63 \times 10^4\) g/min; and (III) microsomes, at \(3.14 \times 10^6\) g/min, according to the procedure described by Randall and Shaw. Fraction III was further fractioned into III-a (sodium deoxycholate-soluble) and III-b (sodium deoxycholate-insoluble) fractions after treatment with 5% sodium deoxycholate. Fractions II, III-a, and III-b were precipitated with cold 30% trichloroacetic acid, washed several times with cold 5% trichloroacetic acid, and then extracted with acid alcohol\(^{12}\) at 37° for 3 hr. The pH of the supernatant was adjusted to 8.5 with concentrated ammonium hydroxide and kept at 4° for 6 hr. Some precipitates were formed and were discarded. The supernatant was dialyzed in 18/32 Visking tubing against running tap water. The dialyze was acidified with glacial acetic acid to a concentration of 1 M and extracted with an equal volume of methylene chloride. The acetic acid fraction was evaporated to near dryness in a rotary evaporator at 37° and lyophilized.

**Gel filtration:** Partially purified pancreatic extracts were chromatographed through a Sephadex G-50 (fine) column (1.5 \(\times\) 86 cm) equilibrated with 1 M acetic acid. Absorption of the fractions at 275 mµmoles was measured in a Cary-15 spectrophotometer. Aliquots were taken for liquid scintillation counting.

**Polyacrylamide gel electrophoresis:** Polyacrylamide gel electrophoresis\(^{13,14}\) was carried out at pH 9.2 in 15% gels containing 7 M urea. The gels were stained with amido black and destained either by electrophoresis in 7% acetic acid or by leaching in 7% acetic acid at 50°. Re-electrophoresis was performed by cutting out the desired band from the gel and rerunning it on a second gel after embedding it in the large-pore gel. Radioactivity of the gel sections was determined by the combustion method described by McEwen.\(^{15}\) The counting efficiency was about 30 to 40%. Pure insulin and single-chain and double-chain proinsulins were obtained as described previously.\(^{16}\)

**Electron microscopy:** Subcellular fractions II and III were suspended in 0.25 M sucrose and centrifuged at 3.14 \(\times\) 10^6 g/min. The pellets were fixed with glutaraldehyde followed by osmium. After dehydration, the tissue blocks were embedded in Epon and Epon-Araldite mixtures. Sections were examined in an RCA EMU 3 g electron microscope.

**Results.—** Gel filtration (Fig. 1) of the partially purified acid alcohol extract of bovine fetal pancreatic slices after incubation with leucine-4,5-H\(^3\) for 2.5 hours showed that a considerable amount of radioactivity was incorporated into a protein fraction (II), possessing the following properties of a proinsulin: (1) immunoreactive with guinea pig anti-bovine insulin serum (Fig. 2), and (2) convertible by trypsin to a smaller protein having the same elution volume as insulin on gel filtration (Fig. 3). Previous experiments have shown that the amount of radioactive leucine incorporated into this fraction increased with the length of the incubation period.\(^{4}\)

Because the technique of gel filtration does not provide sufficient resolution to estimate the amount of radioactivity incorporated, polyacrylamide gel electrophoresis was employed. Polyacrylamide gel electrophoresis of a partially purified

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**Fig. 1.—** Sephadex G-50 gel filtration of acid alcohol-soluble proteins from bovine fetal pancreas after incubation with leucine-4,5-H\(^3\) for 2.5 hr. The arrow indicates the insulin marker.
fetal pancreatic extract showed the presence of insulin and other proteins having electrophoretic mobilities similar to the double-chain proinsulin and the single-chain proinsulin (Fig. 4, C). Electrophoresis of the radioactive extract showed radioactive peaks that coincided with marker proteins of insulin and the two proinsulins (Fig. 4, A). The identity of the radioactive components was further established by reelectrophoresis of the respective bands (Fig. 4, B). The time course of leucine-4,5-H\textsuperscript{3} incorporation into insulin and the two proinsulins was studied, using this reelectrophoresis technique (Fig. 5). The incorporation into these three proteins was inhibited completely by cycloheximide.

Figure 6 shows the electrophoretic patterns of extracts from the three subcellular fractions II, III-a, and III-b. In fraction II, intensely stained bands corresponding to insulin and the double-chain proinsulin were evident. Coelectrophoresis of the extract with marker proteins confirmed the
identification of these proteins. Lightly stained bands corresponding to insulin and the double-chain proinsulin were observed in fractions III-a and III-b. Slices incubated with leucine-4,5-H\textsuperscript{3} were similarly fractionated, and the incorporation of leucine-4,5-H\textsuperscript{3} into the single-chain proinsulin, the double-chain proinsulin, and insulin was examined (Fig. 7). Labeled single-chain proinsulin was present in all fractions. At two hours, fraction III-a and III-b showed radioactive single-chain proinsulin only, whereas fraction II showed all three radioactive proteins. The over-all radioactivity increased after five hours. In fraction III-a an appreciable amount of radioactivity had by then appeared in the double-chain proinsulin and in insulin, whereas in III-b only the single-chain proinsulin was appreciably labeled. In fraction II, however, all three proteins were now highly radioactive after five hours.

Fig. 4.—(A) Polyacrylamide gel electrophoresis of partially purified leucine-4,5-H\textsuperscript{3} labeled acid alcohol-soluble proteins from bovine fetal pancreas. S, D, and I represent the position of marker single-chain proinsulin, double-chain proinsulin, and insulin, respectively. (B) Re-electrophoresis of band S. (C) Electrophoretic pattern of a typical partially purified bovine fetal pancreatic extract.

Fig. 5.—The incorporation of leucine-4,5-H\textsuperscript{3} into single-chain proinsulin, double-chain proinsulin, and insulin in bovine fetal pancreatic slices.
Electron microscopy (Fig. 8) showed that fraction II was rich in granules and fraction III was rich in microsomes.

Discussion and Conclusion.—It is apparent from Figure 5 that a precursor-product relationship exists among the three proteins. Although the quantitative specific radioactivity of these proteins was not determined, it may be concluded from Figures 4, 6, and 7 that the single-chain proinsulin has the highest relative specific radioactivity and insulin the lowest, with the relative specific radioactivity of the double-chain proinsulin falling between these two levels.

As we reported previously, the bovine fetal pancreatic slices incorporated leucine into insulin more slowly than did the isolated pancreatic islets used by Steiner and co-workers. An obvious explanation for this difference could be either the lack of the enzyme(s) required for the conversion of proinsulin to insulin or the inactivity of the enzyme(s) in the bovine fetal pancreas. A recent report that serum from a six-week-old calf contained more proinsulin than insulin lends support to this explanation. A second explanation is that the protease inhibitor that we added to the incubation medium might have inhibited the converting enzyme(s). The actions of protease inhibitors on the conversion process in isolated fish islets and in fat pads have been reported recently.

Our results clearly showed that tritiated leucine was actively incorporated into single-chain proinsulin, double-chain proinsulin, and insulin in slices of bovine fetal pancreas. Steiner and Yip have suggested that a partially cleaved proinsulin found in bovine insulin preparations could be an endogenous intermediate in the biosynthesis of insulin. The incorporation of tritiated leucine...
into the double-chain proinsulin supports this suggestion. The presence of double-chain proinsulin as a major component in the mitochondrial-granule fraction suggested that it may be stored and secreted as insulin is. Recent studies by Rubenstein et al.\textsuperscript{21} and by Yip and Logothetopoulos\textsuperscript{16} demonstrating the existence of a proinsulin in serum would favor this possibility.

Radioactive single-chain proinsulin was present in all three fractions (II, III-a, and III-b) within two hours after the incubation of pancreatic slices with tritiated leucine. Radioactivity was highest in the mitochondrial-granule material, but it was also quite appreciable in the sodium deoxycholate-insoluble microsomal material that represents the ribosomal fraction. However, radioactive double-chain proinsulin and insulin were present in only the mitochondrial fraction at two hours and were virtually absent from the ribosomal fraction (III-b). We therefore offer the speculation that a single-chain polypeptide proinsulin is synthesized on the ribosomes. After the single-chain polypeptide is transferred to the subcellular membranous organelles and to the storage granules, it is converted first to the double-chain intermediate and then to insulin. This speculation is not at variance with the conclusions of Jamieson and Pal-

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**Fig. 7.**—Incorporation of leucine-4,5-H\textsuperscript{3} into single-chain proinsulin, double-chain proinsulin, and insulin in subcellular fractions: fractions III-a, III-b, and II.
Fig. 8.—Electron micrographs of (A) mitochondrial-granule fraction II and (B) microsomal fraction before sodium deoxycholate treatment. G, granule; M, mitochondrion. 49,500X.

ade^{19, 20} on the possible mode of protein biosynthesis and translocation in exocrine secretory cells of the pancreas.
It should be pointed out that the subcellular fractions obviously contained organelles from both the endocrine and exocrine tissues, even though the endocrine tissue in this case represents more than 40 per cent of the total mass of the pancreas. However, the extraction procedure and the analytical method employed have made it possible to study selectively the biosynthesis of insulin and of the two proinsulins in this heterogeneous tissue preparation.

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20. Ibid., p. 597.