MORPHOLOGICAL AND BIOCHEMICAL
STUDIES OF B CELLS OF FETAL RAT ENDOCRINE
PANCREAS IN ORGAN CULTURE

Evidence for (Pro)Insulin Biosynthesis

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ABSTRACT
Fetal rat pancreases explanted on the 18th day of gestation and maintained in organ culture for 1-10 days were utilized for this series of studies. Ultrastructurally, at the time of explantation, the majority of fetal B cells was sparsely granulated and characterized by numerous free ribosomes and undeveloped rough endoplasmic reticulum (RER) and Golgi complexes. During the culture period, extensive development of the RER and Golgi complexes preceded an increasing accumulation of β-granules. This later increase in the number of β-granules and in the concentration of immunoreactive insulin was paralleled by a reduction of RER and Golgi complex activity. High resolution radioautographic studies of pulse-chase experiment over a 1 hr period demonstrated the shift of silver grains from the elements of the RER, through the Golgi region, and finally to the β-granules. Incubation with 14C-labeled leucine demonstrated the incorporation of radioactivity into molecules possessing the immunological and electrophoretic properties of insulin. These studies indicate that de novo synthesis of (pro)insulin occurs also during culture of fetal rat pancreas explanted relatively late in gestation.

INTRODUCTION
Despite the information available concerning the regulation of insulin biosynthesis in the adult pancreas, knowledge of the ontogenesis of this process is as yet much less complete. From the light microscope observations of organ culture preparations of embryonic or fetal pancreas studied in a number of laboratories (1-22), we now know that pancreatic cells survive and may continue to differentiate under organ culture conditions in vitro for some period of time. Less is known, however, of the ultrastructure of the pancreatic endocrine cells in organ culture (7, 23-25), and this has prompted us to undertake an integrated ultrastructural and biochemical study of insulin biosynthesis in organ culture systems.

Accordingly, the aims of the present work are threefold: firstly, to describe the ultrastructure of B cells of the fetal rat pancreas explanted on the 18th day of gestation and maintained up to 10 days in organ culture; secondly, to trace the sequence of events leading to β-granule formation in cultured fetal B cells with high resolution radio-
autography of cultured explants having been exposed to a pulse of labeled leucine; thirdly, to correlate the ultrastructural findings with biochemical studies dealing with (a) the measurement of pancreatic immunoreactive insulin (IRI) concentrations during culture and (b) the incorporation of labeled leucine into IRI molecules of explants cultured in its presence.

MATERIALS AND METHODS

Culture

Rat fetuses were obtained on the 18th day of gestation by cesarean section of the mother under ether anesthesia. The pancreatic explants were excised and cultured in watch glasses according to Chen (5). The composition of the culture medium was 90% (v/v) Eagle HeLa medium (Difco Laboratories, Inc., Detroit, Mich.), 5% adult rat serum, and 5% fetal calf serum, the explants were cultured in a medium to which L-leucine-U-14C (specific activity 311 mCi/m mole, obtained from The Radiochemical Centre). After centrifugation, the TCA-insoluble material was washed four times with cold TCA. The final precipitate was extracted twice with cold acid-ethanol (26), first during 16 hr at 4°C, then again during 4 hr at 4°C. The clear supernatants of both extractions were pooled; ethanol, TCA, and the remaining free L-leucine-U-14C were removed by passage through a Sephadex LH-20 column (1.5 X 15 cm) eluted with 1 N acetic acid. The fractions eluted with the void volume were pooled and concentrated by evaporation. They were redissolved in 1 N acetic acid and passed through a Sephadex G-50 column (1 X 50 cm) again in 1 N acetic acid. The radioactivity was measured in a 0.1 ml sample of each fraction after mixing in 20 ml scintillation fluid (27). The IRI content of another sample was measured after appropriate dilution in phosphate buffer (pH 7.4) containing 0.2% BSA. The fractions in which IRI activity was measured were pooled, evaporated to dryness, and reconstituted in 0.01 N HCl. This fraction was analyzed by electrophoresis in 15% polyacrylamide gel (28) in Tris-glycine buffer at pH 8.9. The gel was cut into 1 mm slices which were extracted in phosphate buffer (pH 7.4) containing 0.2% BSA. Both radioactivity and IRI content were then measured in a sample of each extract.

Extraction of Radioactive IRI

At various intervals after exposure to L-leucine-U14C, the explants were homogenized in cold 6% trichloroacetic acid (TCA). After centrifugation, the TCA-insoluble material was washed four times with cold TCA. The final precipitate was extracted twice with cold acid-ethanol (26), first during 16 hr at 4°C, then again during 4 hr at 4°C. The clear supernatants of both extractions were pooled; ethanol, TCA, and the remaining free L-leucine-U14C were removed by passage through a Sephadex LH-20 column (1.5 X 15 cm) eluted with 1 N acetic acid. The fractions eluted with the void volume were pooled and concentrated by evaporation. They were redissolved in 1 N acetic acid and passed through a Sephadex G-50 column (1 X 50 cm) again in 1 N acetic acid. The radioactivity was measured in a 0.1 ml sample of each fraction after mixing in 20 ml scintillation fluid (27). The IRI content of another sample was measured after appropriate dilution in phosphate buffer (pH 7.4) containing 0.2% BSA. The fractions in which IRI activity was measured were pooled, evaporated to dryness, and reconstituted in 0.01 N HCl. This fraction was analyzed by electrophoresis in 15% polyacrylamide gel (28) in Tris-glycine buffer at pH 8.9. The gel was cut into 1 mm slices which were extracted in phosphate buffer (pH 7.4) containing 0.2% BSA. Both radioactivity and IRI content were then measured in a sample of each extract.

Extraction and Measurement of Protein and Pancreatic IRI

For measurement of IRI content, explants were homogenized twice in cold acid-ethanol as described above. The supernatants of these two extractions were pooled and stored at -20°C until assayed. Protein was measured in the acid-ethanol-insoluble material by the method of Lowry et al. (29). In this tissue, the acid-ethanol-insoluble material represented, quite reproducibly, between 60 and 70% of the total protein content of the tissue, and the values obtained are subsequently referred to as "protein content."
Immunoreactive insulin (IRI) was measured by the double antibody method of Hales and Randle (30), using centrifugation for the separation of the antibody-bound IRI according to Morgan and Lazarow (31). The antisera used in this assay consisted of an anti-beef insulin guinea pig serum and an anti-guinea pig γ-globulin rabbit serum. Pork insulin₁²⁻¹²⁵I and crystalline rat insulin₃ were used as tracer and standard, respectively. This assay allows for the accurate measurement of 0.25 µg (4 µ.units) rat insulin/ml. The cross-reactivity of the anti-insulin serum toward rat proinsulin was estimated by comparing the amounts of labeled insulin displaced from the antibody by equivalent amounts (on a weight basis) of either rat β component, which has been shown to be relatively rich in proinsulin (32), or of crystalline rat insulin. From the data shown in Fig. 1, it has been calculated that 1.5 to two times more β component than insulin were needed to displace radioactive insulin from the antibody. These data indicate that the affinity of this antibody for rat β component was approximately 50-60% that for rat insulin.

Preparation of Tissue for Electron Microscopy

The tissues were fixed in 2.5% cold glutaraldehyde in phosphate buffer, pH 7.6 (33), postfixed in 1% cold osmium tetroxide in the same buffer (33), dehydrated in cold ethanol, and embedded in Epon (34). Sections 0.5–1.0 µ thick were affixed to glass slides and first examined by phase-contrast microscopy. Contiguous thin sections were then cut with glass knives using Porter Blum MT-2 or LKB microtomes, mounted on carbon-coated grids, stained with lead alone, prepared according to Karnovsky (35), and examined with Zeiss EM 9 and Philips EM 300 electron microscopes.

Radioautographic Techniques

High resolution radioautography was performed as described by Caro and van Tubergen (36). Cultured explants were fixed, embedded, and sectioned as described in the previous paragraph. Thin sections were mounted on carbon-coated grids, on which an Ilford L-4 emulsion was applied by the loop method (36). Exposure times varied from 3 to 6 wk. The sections were developed with either Microdol X (Eastman Kodak Co., Rochester, N. Y.) or a “physical” developer (36). They were then stained with uranyl acetate and lead citrate. The distribution of silver grains was determined on photographs of randomly selected fields over five cellular compartments, namely (a) rough endoplasmic reticulum and cytoplasm, (b) Golgi complex, (c) secretory β-granules, (d) mitochondria, and (e) nuclei. When more than 50% of the grain was superimposed upon one of these organelles, it was assumed to be associated with it. A total number of 224–433 grains was counted at each time interval.

RESULTS

Ultrastructure of B Cell before and during Culture

On the 18th day of gestation, the majority of islet cells appeared virtually nongranulated. Granulated cells were in small number and easily identified as A cells (glucagon-producing cells) and B cells (insulin-producing cells) (24). B cells decisively represented the larger number of granulated islet cells. The number of β-granules in each cell was generally rather low (Fig. 2). How-

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₁ The anti-beef insulin antiserum was a gift from Dr. P. H. Wright, Indiana University, Indianapolis.

₂ Crystalline rat insulin (15 units/mg) and rat β component were generously provided by Dr. J. Schlichtkrull, Novo Research Institute, Copenhagen, Denmark.
Figure 2  Fetal rat pancreas on the 18th day of gestation. Low power electron micrograph of a section through an islet. Most cells are virtually nongranulated (NG) or poorly granulated (G1); their cytoplasm contains numerous free ribosomal particles but few elements of rough-surfaced endoplasmic reticulum. Parts of well granulated B cells (G2) can also be seen. C, capillary. \( \times \) 8000.
ever, in some cells, preferentially located near the capillaries, granulation was more abundant (Fig. 2). The most conspicuous features of the non-granulated cells and of the poorly granulated B cells were numerous free ribosomes and polyribosomes, relatively few profiles of rough endoplasmic reticulum (RER), and a structurally poorly elaborated Golgi complex. Only rarely were a relatively prominent RER and a clearly defined Golgi complex observed.

During the first day of culture, the B cells retained essentially unchanged morphological characteristics (Fig. 3). As the duration of culture progressed, however, extensive development of both RER (Fig. 4) and Golgi complex (Fig. 5) was observed in an increasing number of B cells, followed in time by the progressive accumulation of secretory granules (Fig. 8). Nevertheless, sparsely granulated B cells could still be observed throughout the culture period.

The RER most often consisted of groups of several elongated and parallel cisternae (Fig. 4) which, as secretory granules accumulated in the cytoplasm, were predominantly located at the periphery of the cell. In general, elements of the RER surrounding the Golgi complex tended to lose their ribosomes and appeared as partly smooth-, partly rough-surfaced membranes (Fig. 6) likely corresponding to the "junction elements" described in exocrine pancreatic cells (37). Between these junction elements and the Golgi complex, clusters of microvesicles were noted. These microvesicles seemed to proceed from buds of smooth-surfaced areas of the RER (Fig. 6). Similar clusters of smooth-surfaced microvesicles have previously been described in pancreatic exocrine cells in which they have been considered as "shuttle carriers" between the RER and the Golgi complex (38, 39).

The Golgi complex, usually located in the perinuclear area, was often large and consisted of stacks of several curved cisternae, more or less dilated. In many instances, there was evidence for the presence of maturing secretory granules in the Golgi cisternae (Fig. 5, 7). Whenever B cells appeared filled with secretory granules, the RER (Fig. 9) and Golgi complex tended to be reduced, both in size and in structural complexity.

Radioautographic Studies

Although, at each time interval after the labeling pulse, silver grains were found over most cell components, the distribution of the grains varied notably with time, as shown in Table I. Thus, at the end of the labeling, about 79% of the grains were present over either RER or cytoplasm (Figs. 10, 11, 14). 15 min later, the percentage of the grains associated with RER and cytoplasm had markedly decreased (37%) while that of the grains over the Golgi complex had considerably increased (42%) (Fig. 12). Between 15 and 30 min, the distribution of the grains did not change strikingly (Fig. 15). The highest percentage of grains over the secretory granules occurred at 60 min (44%) (Figs. 13, 16, 17), while labeling of the Golgi complex had diminished to 13%. At all times, the labeling of mitochondria and nuclei remained relatively constant and the percentage of background grains over epoxy resin areas devoid of tissue remained negligible.

IRI Content of Fetal Rat Pancreas during the Culture Period

As shown in Table II, pancreatic wet weight decreased progressively beyond the fourth day of the culture period, while the ethanol-insoluble protein content of the explant remained relatively stable throughout 10 days. Evidently, therefore, the fetal explants did not grow significantly during the culture period. Pancreatic IRI concentration, however, increased considerably as a function of time up to 10 days of culture: on the 18th day of gestation, pancreatic IRI concentration amounted to 0.16 milliunit/mg wet weight and 3.68 milliunits/mg protein. During the first 2 days of culture, it increased about 10-fold to reach values of 2.27 milliunits/mg wet weight and 31.70 milliunits/mg protein. From the 2nd up to the 10th day of culture, pancreatic IRI concentration continued to increase, though at a slower rate, to reach 13.0 milliunits/mg wet weight, and 109.6 milliunits/mg protein, values which represent an over-all increase of 80- and 30-fold, respectively.

Biochemical Evidence for IRI Biosynthesis

Fig. 18 illustrates the elution patterns of radioactivity and IRI activity from Sephadex G-50 column of acid-ethanol-soluble protein extracted from explants cultured for 2, 4, 6, and 8 days in a medium containing leucine-U-14C throughout. At each interval, there was a major early peak of radioactivity containing no detectable IRI activi-
FIGURES 5–7  Fetal rat pancreas explanted on the 18th day of gestation and cultured 2 days. Fig. 5: shows a large juxtanuclear Golgi complex and many profiles of granules undergoing formation (arrows). × 17,200. Fig. 6: shows the characteristic polarization of the partly smooth wall of Golgi-near rough-surfaced element of endoplasmic reticulum towards the Golgi complex (G). The arrow indicates an area where the wall of ER cisternae appears to be budding towards the Golgi complex. The contents of the bud indicated exhibit electron opacity similar to that of the neighbouring microvesicles. × 37,000. Fig. 7: shows a secretory granule arising in the inner Golgi cisternae (arrow) × 37,000.

ity. This peak corresponded to the excluded large molecules. After 2 days of culture, no significant radioactivity could be detected in the region where IRI was eluted (fractions 25–35). After 4 days of culture, however, a shoulder of radioactivity appeared in that region, and after 6 and 8 days of culture, a definite second peak of radioactivity was observed, which coincided with the IRI peak and which also increased as a function of culture time and of total IRI activity. When fractions 25–35

FIGURE 3  Fetal rat pancreas explanted on the 18th day of gestation and cultured 1 day. Part of B cells containing numerous polyribosomes throughout the cytoplasm, a few secretory granules (sg), and some elements of rough-surfaced endoplasmic reticulum (er). Nucleus (N). × 23,000.

FIGURE 4  Fetal rat pancreas explanted on the 18th day of gestation and cultured 2 days. Areas of the cytoplasm of a B cell showing abundant rough-surfaced endoplasmic reticulum (er) and relatively few secretory granules, some of which (arrows) are located in the Golgi region (G), probably undergoing maturation. × 28,000.

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were pooled and analyzed by polyacrylamide gel electrophoresis (Fig. 19), two major peaks of IRI activity with $R_f$ values corresponding to those of the two rat insulins were obtained. These peaks again coincided exactly with the two radioactivity peaks.

DISCUSSION

During the culture of fetal rat pancreas explants, perhaps the most striking ultrastructural feature observed was the gradual shift in the relative prominence of the different organelles concerned with protein synthesis and the packaging of secretory products. A marked increase in RER and in the complexity of the Golgi complex came first and preceded in the cultured B cells the definite and continuous yet gradual accumulation of secretory $\beta$-granules. The morphological evidence for peak activity of RER and Golgi complex was further associated with active budding and accumulation of microvesicles, predominantly in the region intermediate between RER and Golgi cisternae. Further, the evidence strongly favors an inverse relationship existing at any given time between the development of the RER and of the Golgi complex, on the one hand, and the number of $\beta$-granules
Figure 9  Fetal rat pancreas explanted on the 18th day of gestation and cultured 8 days. Part of a B cell filled with secretory granules. Note the relative sparsity of elements of rough endoplasmic reticulum (arrows).  $\times$ 37,000.
TABLE I

Distribution of Radioautographic Grains over Cell Components

| Component                                | % of radioautographic grains | Pulse (5 min) | Chase incubation |
|------------------------------------------|------------------------------|---------------|------------------|
| Rough endoplasmic reticulum and cytoplasm|                             | 78.7          | 36.6 33.3 25.8   |
| Golgi complex                            |                             | 4.5           | 41.7 37.4 12.8   |
| Secretory granules                       |                             | 5.2           | 5.1 12.8 44.3    |
| Mitochondria                             |                             | 3.1           | 3.4 5.4 5.5      |
| Nuclei                                   |                             | 8.5           | 13.2 11.1 11.6   |
| No. of grains counted                    |                             | 224           | 205 433 342      |

Stored in any one B cell, on the other, as previously reported for B cells from adult animals (40, 41). Because reports regarding the role played by the Golgi complex in the formation of β-granules are still conflicting, the sections through the Golgi areas were scrutinized with special care: maturing granules were frequently observed in Golgi cisternae, an observation supporting previous studies in vivo suggesting that packaging of secretory β-granules takes place here (41-48). Conversely, we have been consistently unable to find evidence for the direct formation of β-granules from cisternae of the RER, bypassing the Golgi complex, as suggested by others (49-55).

The radioautographic studies were performed in order to obtain more direct and dynamic evidence concerning the role of various cytoplasmic organelles during the active storage of insulin. In order to enhance maximally (pro)insulin biosynthesis in these experiments, the cultured explants had been incubated for 15 min before the labeling pulse in a medium with a relatively high (16.5 mM) glucose concentration known to allow for optimal increments in explant IRI content during culture (18). Moreover, in adult B cells also, a high glucose concentration is known to increase the rate of incorporation of labeled amino acids into insulin (56). Under these conditions, it is reasonable to assume that a large number of the radioautographic grains reflects incorporation of radioactivity from the labeled amino acid precursor into exportable proteins, presumably (pro)insulin.

Immediately after the labeling with leucine-3H, the majority of the silver grains was found over elements of the RER, indicating that (pro)insulin biosynthesis in organ culture of fetal B cells may be initiated at the level of the RER as earlier suggested for adult mammalian cells by Lacy (49) and confirmed by Bauer et al. (57) from studies of the radioactivity distribution in subcellular fractions from goosefish islets incubated with labeled leucine. After 15 and 30 min of “chase” with unlabeled leucine, predominant radioactivity clearly shifted from the RER to the Golgi complex, indicating transfer of newly synthesized protein to this organelle, and after 60 min, radioactivity was further displaced from the Golgi complex to the β-granules.

The heterogeneity of the cell population of our cultured explants (18) has prevented us as yet from isolating fractions of B cells with any degree of confidence in the identity of the fractions obtained. However, the results of the present radioautographic studies clearly indicate that the morpho-

Figures 10–13 Electron microscope radioautograph of B cells at the end of pulse labeling for 5 min (Figs. 10, 11), and after additional 15 min (Fig. 12) and 60 min (Fig. 13) incubation in chase medium. Development in Microdil-X. Fig. 10: the radioautographic grains appear over the rough-surfaced elements of the endoplasmic reticulum or the cytoplasm between them. X 21,000. Fig. 11: in the well granulated B cell the radioautographic grains are located over elements of cytoplasm between the secretory granules. X 12,000. Fig. 12: shows most of the grains overlying the elements of the Golgi complex. X 14,000. Fig. 13: shows radioautographic grains principally located over secretory granules. X 24,000.
Figures 14–17  Electron microscope radiosautograph of B cells at the end of pulse labeling for 5 min (Fig. 14), and after additional 30 min (Fig. 15) and 60 min (Figs. 16, 17) incubation in chase medium. Development in fine grain physical developer (36). Fig. 14: although a few grains overlie mitochondria and nucleus, almost all of them are localized over rough-surfaced elements of the endoplasmic reticulum or the cytoplasm. Secretory granules are practically devoid of label. X 20,500. Fig. 15: almost all grains overlie elements of the Golgi complex. X 38,000. Fig. 16: notice the good localization of the grains with respect to the secretory granules. Some grains overlie elements of the Golgi complex and the surrounding cytoplasm. X 35,500. Fig. 17: shows the accumulation of grains in numerous secretory granules. X 25,500.
TABLE II

| Days of culture | Wet weight (mg per pancreas) | Protein content (mg per pancreas) | IRI concentration |
|-----------------|-------------------------------|-----------------------------------|-------------------|
|                 | wet weight                    | protein content                   |                   |
| 0               | 0.90 ± 0.07 (26)              | 0.047 ± 0.003 (26)                | 0.16 ± 0.04 (26)  |
| 2               | 0.70 ± 0.12 (10)              | 0.045 ± 0.006 (10)                | 2.27 ± 0.64 (10)  |
| 4               | 0.65 ± 0.09 (10)              | 0.053 ± 0.005 (10)                | 3.02 ± 0.27 (10)  |
| 6               | 0.50 ± 0.11 (10)              | 0.051 ± 0.004 (10)                | 5.03 ± 0.33 (10)  |
| 8               | 0.54 ± 0.09 (10)              | 0.051 ± 0.006 (10)                | 8.53 ± 1.00 (10)  |
| 10              | 0.51 ± 0.09 (16)              | 0.053 ± 0.006 (16)                | 13.03 ± 2.02 (16) |

Each figure represents the mean ± SEM of the number of observations indicated in parentheses.

Figure 18 Radioactivity-14C (open circles) and IRI activity (closed circles) patterns of acid-ethanol-soluble material from cultured fetal pancreas after elution on a Sephadex G-50 column (fractions of 1 ml). A, B, C, and D refer to explants cultured in a medium containing leucine-14C over periods of 2, 4, 6, and 8 days, respectively.

Figure 19 Radioactivity-14C (open circles) and IRI activity (closed circles) patterns after electrophoresis on polyacrylamide gel of pooled fractions Nos. 25-35 from a Sephadex G-50 column. In this experiment, fetal explants were cultured over 6 days in a medium containing leucine-14C.

The logical sequence concerned with the intracellular movements of newly synthesized protein to its storage site in B cells of cultured fetal explants is quite similar to that recently reported by Howell et al. (58) for adult pancreatic B cells. Also, the sequence proposed is in general agreement with that initially described for exocrine pancreatic cells (38, 59, 60). It would therefore seem likely that, both in cultured explants and in adult islets, protein synthesis takes place within the RER from which the newly synthesized material is ferried, at least in part, to the Golgi complex where it is condensed into secretory granules.

The ultrastructural evidence for active protein, presumably (pro)insulin, synthesis receives strong support from the measurement of pancreatic IRI and from the studies dealing with the rate of incorporation of leucine-U-14C into IRI molecules.
Pancreatic IRI concentration increased, over a 10 day culture period, by a factor of 80- or 30-fold when referred to unit weight or unit protein, respectively. Although it is recognized that some of this increase could be the result of transformation of less immunoreactive proinsulin into more immunoreactive insulin (61), the relative affinities for rat insulin and the proinsulin-rich rat b component of the antiinsulin serum used in our immunoassay procedure would not allow for more than two- or threefold increment on this basis alone. Moreover, labeled leucine is incorporated into IRI molecules of cultured explants at a rate roughly parallel to the rise in IRI concentration, and the polyacrylamide gel electrophoresis studies establish that most of the radioactivity coincides with insulin, not with proinsulin bands. Together with the morphological and radioautographic data previously discussed, these biochemical findings clearly establish that active de novo (pro)insulin biosynthesis and storage take place in B cells of cultured fetal explants.

Clark (61) had studied (pro)insulin biosynthesis in cultured pancreas explants principally obtained much earlier in the gestation period. Clark also found a rapid increase in IRI concentration during development after the 15th day of gestation; he suggested, however, that this increase resulted almost exclusively from the conversion of precursor (proinsulin) molecules which had been synthesized between the 11th and 14th days of gestation. The results of our morphological, radioautographic, and biochemical studies, derived from explants obtained on the 18th day of gestation, do not support Clark's conjecture.

We have described elsewhere in some detail the biochemical and morphological characteristics of IRI release from fetal rat explants (25, 62-65). The principal feature of fetal as compared to postnatal or adult tissue is that of relative insensitivity to a number of physiological and pharmacological stimuli administered individually, "normal" adult-type sensitivity being restored either by cumulation of several stimuli or by the addition of inhibitors of phosphodiesterase such as methylxanthines (25, 63-65). The typically fetal type of IRI release response is retained throughout organ culture periods.

The morphological equivalents of IRI release, however, at no time allowed for a differentiation of fetal from adult tissue.

Taken as a whole, both our morphological and biochemical studies suggest that, during organ culture, insulin biosynthesis progressed from the characteristics of fetal (18 days of gestation) tissue to characteristics indistinguishable from those of postnatal or adult tissue. Insulin release, however, retained the fetal response pattern throughout culture for as long as 8 days (data published elsewhere), yet an ultrastructural equivalent of the fetal release state could not be found.

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