Proteins Synthesized and Secreted during Rat Pancreatic Development

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ABSTRACT The synthesis and secretion of proteins during development of the pancreas was analyzed using two-dimensional gel electrophoresis. The pattern of synthesis of the total proteins of the pancreas was found to change very little from 14 to 18 d gestation. In addition, the protein synthetic pattern of the embryonic pancreas was very similar to the protein patterns of several other embryonic tissues (gut, lung, and mesenchyme). Between 18 d gestation and the adult stage, the synthesis of the majority of protein species fades as the synthesis of the secretory (pro)enzymes becomes dominant. Thus, the terminal differentiation of the pancreas appears to involve the dominant expression of a limited set of genes (coding, in part, for the digestive [pro]enzymes) while the pattern of expression of the remaining domain remains relatively unchanged. Many of the secretory (pro)enzymes were identified and their synthesis during development was monitored. The synthesis of several secretory proteins was detected between 15 and 18 d gestation (e.g., amylase and chymotrypsinogen), whereas the synthesis of others was not detected until after 18 d gestation (i.e., trypsinogen, ribonuclease, proelastase, and lipase). Between 18 d gestation and the adult stage, the synthesis of the digestive (pro)enzymes increases to >90% of pancreatic protein synthesis. The secretion of digestive (pro)enzymes was detected as early as 15 d gestation. The selective release of a second set of proteins was detected in the early embryo. These proteins are not detected in the adult pancreas or in zymogen granules but are also released by several other embryonic tissues. The function of this set of proteins is unknown.

Pancreatic differentiation has been extensively studied at both morphological (18, 19) and biochemical levels (5, 14, 23, 25). Normal development requires interaction between epithelial and mesenchymal tissues (8, 25). Early investigations suggested that pancreatic differentiation is a multiphasic process (4, 21, 23). In the rat, the formation of the pancreatic rudiment from the gut at ~11 d gestation is coupled to the appearance of very low levels of exocrine proteins and insulin (the primary transition). During the next 3–4 d, acinar structures form and the low levels of exocrine secretory proteins and insulin are maintained ("protodifferentiated state") (22, 23). A second differentiative transition is detectable beginning at 14–15 d gestation when there is a rapid increase in rough endoplasmic reticulum, a 10^3- to 10^4-fold increase in the accumulation of the specific exocrine enzymes and insulin, coupled with the appearance of zymogen granules in acinar cells and β granules in B cells (4, 21, 23). The messenger RNAs coding for the exocrine proteins accumulate in a parallel fashion but slightly preceding the secretory proteins (10, 11, 20).

We have now used two-dimensional gel electrophoresis to monitor protein synthesis and secretion during development of the embryonic pancreas. This method allows analysis of both the pancreas-specific products (the secretory [pro]enzymes) and the synthetic patterns of the total proteins. Several of the secretory proteins have been identified and their synthesis and accumulation during the secondary transition (14, 23, 26) have been confirmed. The secretion of these proteins at embryonic stages has been demonstrated. We further demonstrate that while the synthesis of the secretory proteins increases dramatically between 14 and 18 d gestation, very little change occurs in the synthetic pattern of the total proteins. Furthermore, at early stages of differentiation, the protein synthetic patterns of the pancreas and various other tissues are strikingly similar. These studies suggest that pancreatic differentiation involves

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the synthesis of a (small) subset of prominent proteins, while the expression of the majority of the proteins remains unchanged.

MATERIALS AND METHODS

In Vitro Labeling of Tissues

Adult or embryonic organs were dissected from Sprague-Dawley rats. Pregnancies were timed and dissections were performed as described previously (18). Freshly dissected adult pancreatic lobules, intact embryonic pancreases and lungs, sections of embryonic gut and individual lobes of embryonic liver were incubated in a HEPES-buffered Krebs-Ringer's solution (28) supplemented with minimal essential medium (MEM) amino acids (Grand Island Biological Co., Santa Clara, Calif.) (without leucine), 2 mM glutamine, 1 g/liter glucose, 50 μg/ml bovine serum albumin, 0.1 mg/ml soybean trypsin inhibitor, 100 U/ml penicillin, 100 μg/ml streptomycin, and 125 μCi/ml [^{3}H]leucine (60 Ci/mmol, New England Nuclear, Boston, Mass.). In some cases the labeling was done in supplemented MEM (GIBCO) (without leucine) without HEPES-buffered Krebs-Ringer's. Typically, incubations included, respectively, 30 adult lobules, 10–40 embryonic pancreases (depending on age), 20 16-d embryonic lungs, 10 16-day embryonic liver lobes and 20 ~1-cm-long pieces of 16-d embryonic gut. In cases in which released proteases were to be assayed, soybean trypsin inhibitor was omitted and bovine serum albumin was increased to 100 μg/ml. The tissues were incubated for 6 h at 37°C with shaking. A continuous stream of 100% oxygen was passed over the HEPES-buffered medium, and 95% oxygen, 5% carbon dioxide was used with the MEM. 14-d pancreases cultured as described previously (18) were labeled as described above.

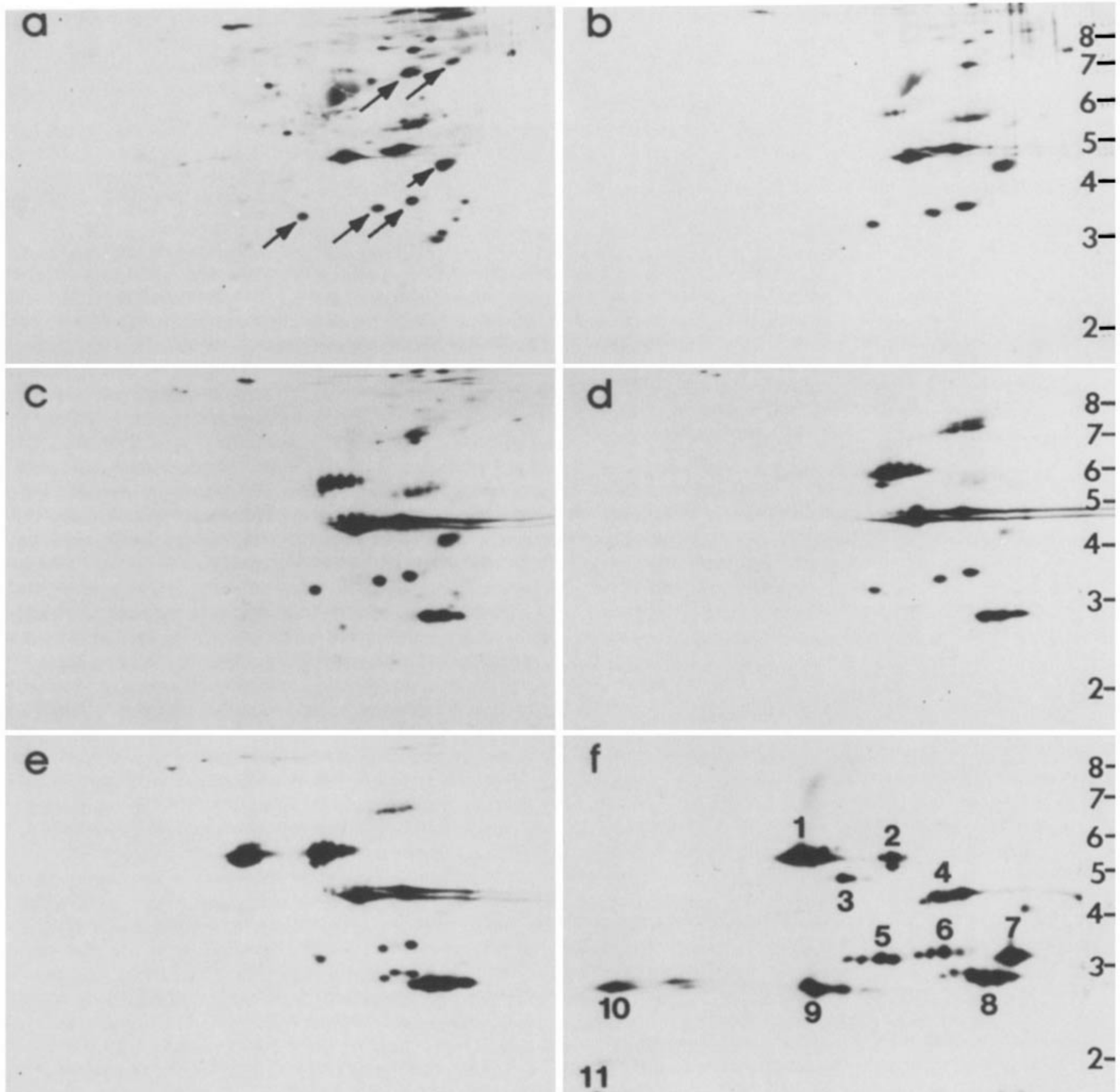


FIGURE 1 Two-dimensional gel electrophoretic analysis of proteins released by embryonic and adult pancreases. Nonequilibrium pH gradient electrophoresis is in the horizontal direction with the basic end at the left and the acidic end at the right. SDS electrophoresis is in the vertical direction. *a*, 14-d released proteins; *b*, 15-d; *c*, 16-d; *d*, 17-d; *e*, 18-d; *f*, adult. Arrows indicate proteins released by embryonic pancreas as well as several other embryonic organs (see text) but not by adult pancreas. The scale at the right indicates molecular weight × 10^{-4} based on the migration of identified zymogens of known molecular weight (ribonuclease, 13,000; chymotrypsinogen, 26,000; procarboxypeptidase B, 45,000; and amylase, 54,000).

Two-dimensional Gel Electrophoresis

The labeling media were centrifuged for 10 min at 16,000 *g* to remove tissue debris and dialyzed exhaustively against distilled water at 4°C. The dialyzed samples were frozen, lyophilized, and redissolved in small volumes of NEPHGE (nonequilibrium pH gradient gel electrophoresis) sample buffer (9.5 M urea, 2% pH 3.5–10 ampholytes (LKB, Uppsala, Sweden), 5% β-mercaptoethanol, and 2% Nonidet P-40 [NP-40]) (17). Labeled embryonic organs and adult pancreatic lobules were washed three times with Earle's balanced salt solution (GIBCO) and placed in sonication buffer (0.01 M Tris, 5 mM $CaCl_2$, 0.1 mg/ml soybean trypsin inhibitor, 40 μg/ml micrococcal nuclease) (16). The organs were homogenized with 10 strokes of a glass Teflon homogenizer, sonicated, and incubated for 5 min at room temperature. The homogenates were then made up to 9 M urea and mixed with an equal volume of NEPHGE sample buffer. The samples (up to 25 μl) were analyzed by NEPHGE-SDS two-dimensional gel electrophoresis as described by O'Farrell et al. (17). The first-dimension electrophoresis was performed at 400 V for 4 h. The gels were extruded from the tubes and equilibrated for 2 h in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.063 M Tris, pH 6.8). A 12% acrylamide resolving gel and a 3.75% stacking gel were used for the second-dimension electrophoresis. Finished gels were then prepared for fluorography as described by Bonner and Lasky (2) and autoradiographed on Kodak X-Omat film at −70°C.

Flat-bed Isoelectric Focusing

Flat-bed isoelectric focusing was performed in a 20-cm × 25-cm bed of Bio-Gel P-60 (Bio-Rad, Richmond, Calif.) containing 2% pH 3.5–10 ampholytes (LKB). Buffer strips at each end of the Bio-Gel matrix consisted of 3 0.8-cm × 20-cm strips of Whatman 3MM paper (Whatman Inc., Clifton, N. J.) soaked in 2% ampholytes. Identical strips soaked in either 1 M H_2SO_4 (anode) or 2 M NaOH (cathode) were placed directly on top of the buffer strips. Platinum electrodes were laid across the electrode strips. The system was prerun at 10–20 mA for 2 h. The samples were spotted onto the Bio-Gel matrix and run at 400 V for 24 h. Sample lanes were cut into 0.5-cm sections, suspended in 0.1 M NaCl, and analyzed for radioactivity and enzyme activity.

Enzyme Activation and Assay

Trypsinogen, chymotrypsinogen, and procarboxypeptidase A and B were activated as previously described (26, 28). Trypsin and chymotrypsin activities were measured by the method of Hummel (12). carboxypeptidase B by the method of Folk et al. (7), amylase by the method of Bernfield (1), and ribonuclease by the method of Kalnitsky et al. (13).

RESULTS

Proteins Secreted by the Adult Rat Pancreas

Fig. 1*f* shows the proteins secreted by adult pancreatic lobules in culture. At least 12 major proteins are resolved by two-dimensional gel electrophoresis (numbered *1–12*). These proteins are the exocrine enzymes or proenzymes found in zymogen granules and are secreted in response to secretagogues (9, 28, 29). Several of the proteins (e.g., 2, 5, 6, 8) appear as multiple spots in the horizontal dimension of the gels. Although it is in principle possible that these are a result of intrinsic artifacts, numerous controls carried out by others (16, 17) and by ourselves (see studies in protein 2, below) suggest this is not the case. These spots are probably molecules with single-unit charge differences caused by modification such as glycosylation and phosphorylation (16). In this report such heterogeneous sets are considered single proteins.

Several of the adult secretory proteins were identified by use of preparative flat-bed isoelectric focusing. Unlabeled adult zymogen granule contents were run in parallel and used for some identifications. Tartakoff et al. (29) have shown that adult secretions and zymogen granule contents are similar (also cf. Figs. 1*f* and 3*a*). The radioactivity and the amylase activity of each fraction of the adult secretions, as well as the location of carboxypeptidase B, chymotrypsin, trypsin (after activation), and ribonuclease activities from the zymogen granule lysate run in parallel, are shown in Fig. 2. Amylase has two main

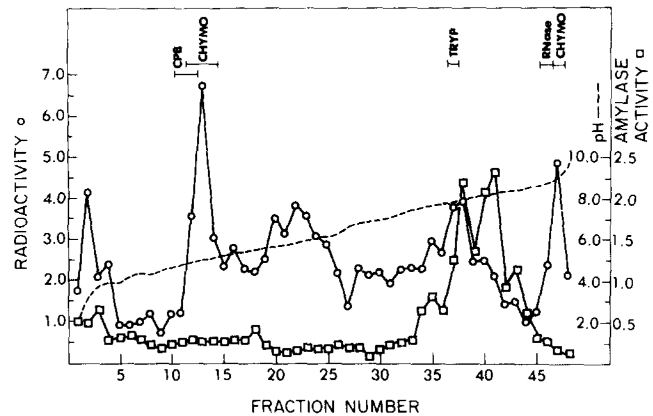


FIGURE 2 Flat-bed isoelectric focusing of adult pancreatic secretory proteins. [^{3}H]leucine-labeled adult pancreatic secretions and unlabeled zymogen granule lysate were run side by side on flat-bed isoelectric focusing (see Materials and Methods). The radioactivity in ^{3}H cpm × 10^{-3} (○), amylase activity in milligrams of maltose per minute per fraction (□), and pH (- - -) of each fraction of the adult secretions is shown. Fractions of the zymogen granule lysate lane that contained ribonuclease (*RNase*), and, after activation, chymotrypsin (*CHYMO*), trypsin (*TRYP*), and carboxypeptidase B (*CPB*) activities are indicated.

isoelectric forms in Fig. 2 that co-migrate as protein *1* in the NEPHGE system (Fig. 1*f*). The identification of protein *1* as amylase was confirmed by immunoprecipitation with rabbit antirat amylase antiserum (results not shown). Spot *4* appears to be a complex of three species of slightly different charge and molecular weight (Fig. 1*f*). Procarboxypeptidase B isolated by flat-bed isoelectric focusing migrates within the spot *4* complex on two-dimensional gel electrophoresis. Two forms of chymotrypsinogen were isolated by flat-bed isoelectric focusing, an acidic form (pI 4.9) and a basic form (pI 9.1). The acidic form runs identically to spot *8* and the basic form appears to be spot *10*.

Trypsinogen migrated with a pI of ~7.8 at the acidic edge of the amylase activity on flat-bed isoelectric focusing. From its isoelectric point as well as its size relative to chymotrypsinogen, we conclude that spot *9* is trypsinogen. Ribonuclease activity exhibited an isoelectric point of 8.8 in flat-bed isoelectric focusing; we conclude spot *11* is ribonuclease on the basis of its isoelectric point and size. Protein *2* has yet to be identified (see below). By comparing the relative migration of the remaining proteins (numbers *3*, *5*, *6*, and *7*) in the SDS dimension to those identified by Scheele (27) in guinea pig pancreatic secretions, it appears that protein *3* is lipase (slightly smaller than amylase) and that proteins *5*, *6*, and/or *7* may be proelastase (slightly larger than chymotrypsinogen). Scheele (27) demonstrated two proelastase forms in guinea pig, differing slightly in molecular weight and isoelectric point.

Proteins Secreted (Released) by Embryonic Rat Pancreases

Pancreases of embryos at 14–18 d gestation were dissected and incubated in a medium containing [^{3}H]leucine as described in Materials and Methods. A subset of the total proteins synthesized (compare with Fig. 5) is released into the medium (Fig. 1*a–e*). At early ages a large number of proteins are released, but with increasing embryonic age, a smaller distinct subset can be recognized. Three groups of proteins accumulate in the medium: (*a*) proteins found in adult secretions and

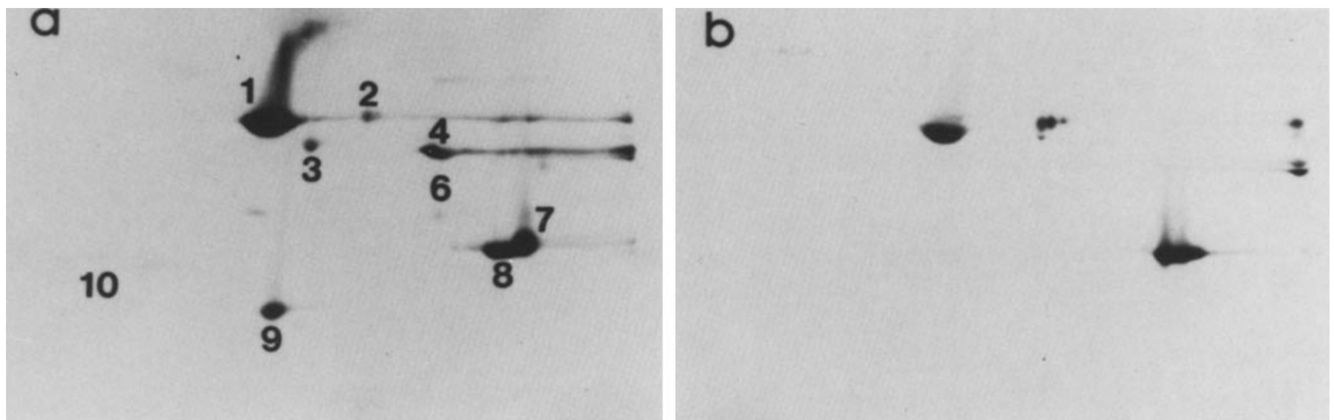


FIGURE 3 Comparison of the proteins of adult and 18-d zymogen granule lysates. [^{3}H]leucine-labeled zymogen granules were isolated and lysed, and the lysate was applied to two-dimensional gel electrophoresis as in Fig. 1. *a*, Adult zymogen granule lysate; *b*, 18-d zymogen granule lysate. Numbered adult proteins correspond to those numbered in Fig. 1 *f*.

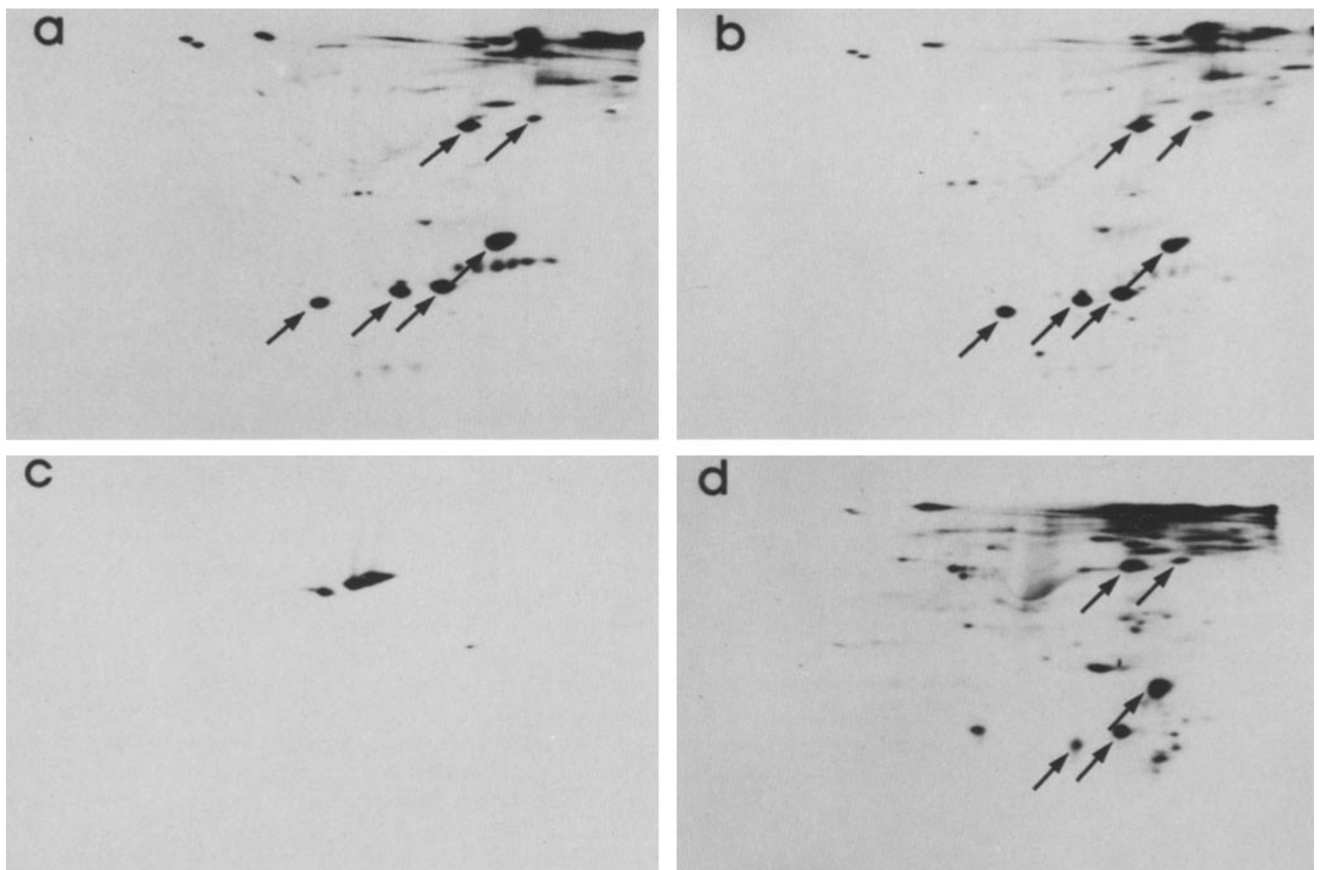
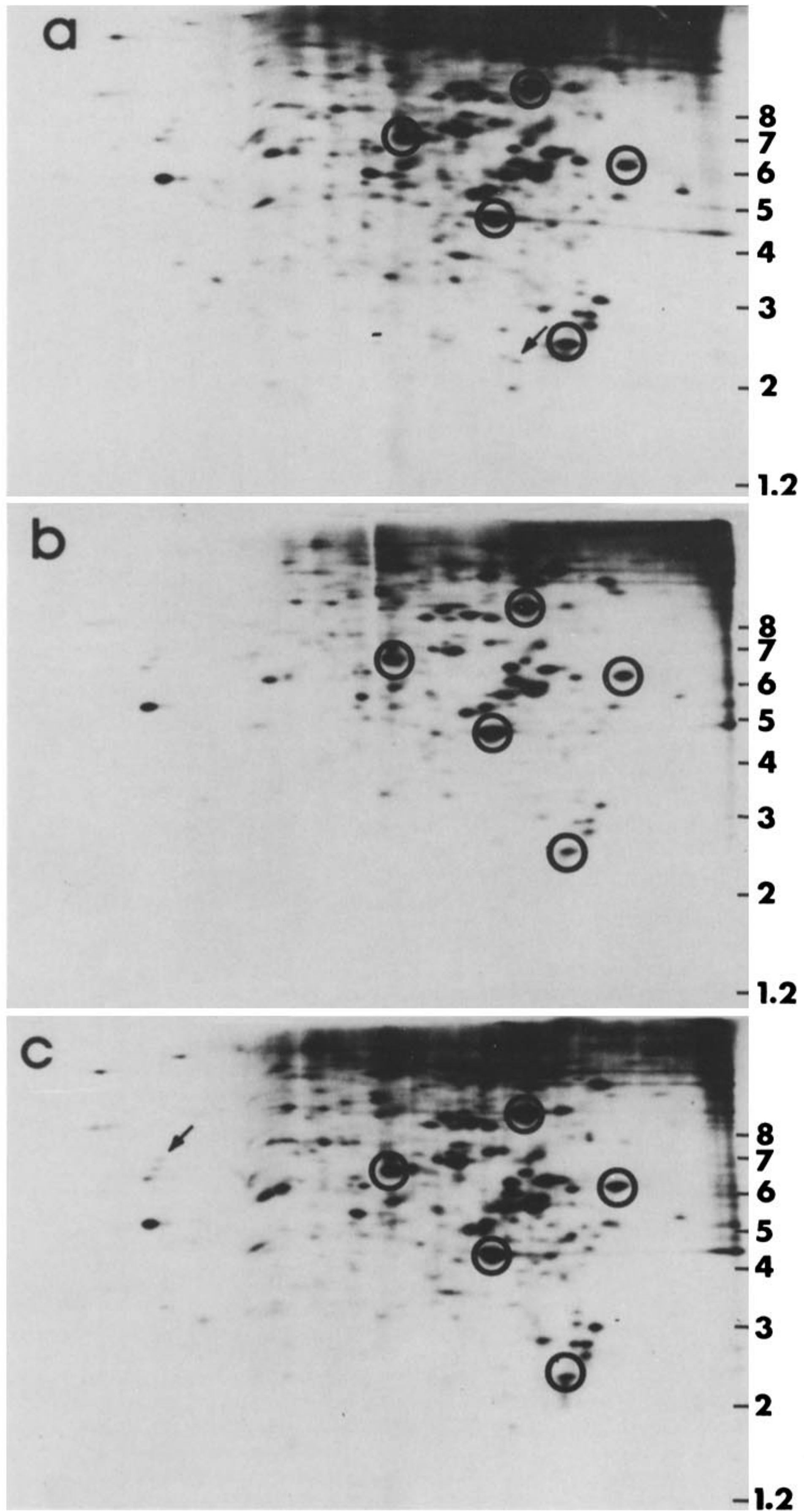


FIGURE 4 Comparison of the proteins released by embryonic gut, lung, liver, and mesenchymal tissue. Electrophoresis was performed as in Fig. 1. *a*, 16-d gut released proteins; *b*, 16-d lung; *c*, 16-d liver; *d*, 14-d mesenchyme. Arrows indicate proteins released in common by embryonic pancreas, gut, lung, and mesenchyme.

zymogen granules, i.e., secretory (pro)enzymes (proteins *1*, *2*, *4*, and *8*) (we term these the secretory subset); (*b*) proteins detected during embryonic development but not detectable in either adult secretions or in gels of total proteins synthesized by the embryonic pancreas (arrows, Fig. 1 *a–e*) (we term these the released subset); and (*c*) those proteins prominent in gels of total cellular proteins (compare with Fig. 5). The latter set is detected primarily at days 14 and 15 and may be the result of cell damage or leakage from the early embryonic cells. None of the secretory (pro)enzymes are detected at 14 d gestation by this analysis. Protein *2* is first detected at day 15, protein *8* (chymotrypsinogen) is first detected at day 16, and protein *1* (amylase) is first detected at day 17. Protein *4* may be secreted at embryonic stages, but it is obscured by at least two other dominant proteins at all stages. Several proteins present in adult secretions are not released at detectable levels by embryonic pancreases of 14–18 d gestation, including proteins *3* (lipase), *7* (proelastase), *9* (trypsinogen), *10* (chymotrypsinogen II), and *11* (ribonuclease). When the proteins released by embryonic pancreases of 20 d gestation were analyzed (data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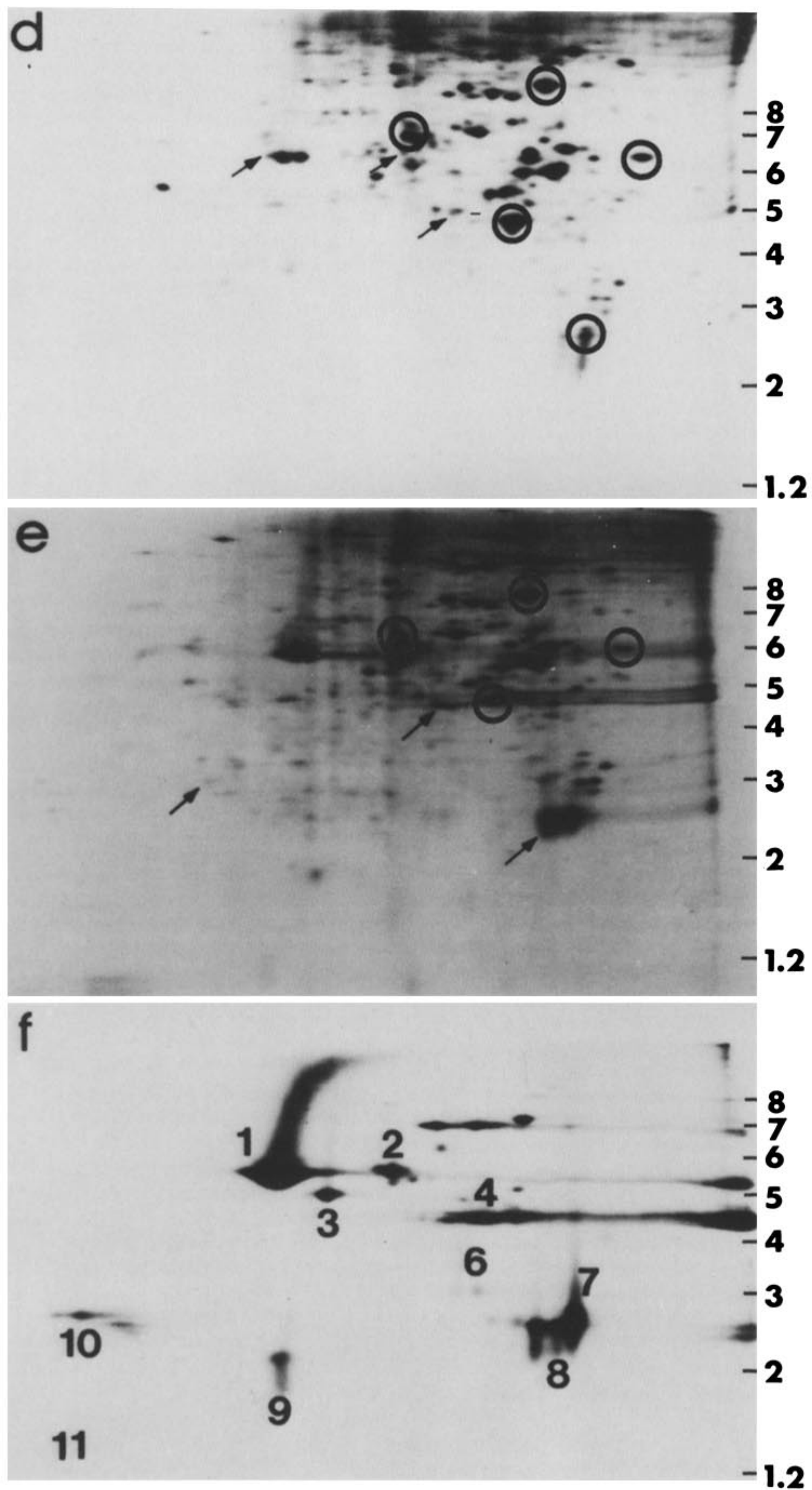

FIGURE 5 Two-dimensional gel electrophoresis of adult and embryonic total pancreas proteins. Electrophoresis was performed as in Fig. 1. *a*, 14-d pancreas proteins; *b*, 15-d; *c*, 16-d; *d*, 17-d; *e*, 18-d; and *f*, adult. Arrows pointing up indicate new proteins that appear between day 14 and day 18 of gestation and are indicated on the first day detectable. Arrows pointing down indicate early pancreas proteins whose synthesis is shut off between day 14 and day 18 of gestation. Circled spots indicate common reference proteins to assist comparison between figures. The scale at the right indicates molecular weight $\times 10^{-4}$ as in Fig. 1.

not shown) proteins *3*, *7*, *9*, *10*, and *11* can be detected at low levels. Between 20 d gestation and the adult stage the secretion of these proteins increases dramatically.

Protein *2*, a member of the secretory subset, has not been identified, but it is present in zymogen granules (18 d and adult; see Fig. 3) as well as in secretory products from pancreases older than 15 d gestation. This component reaches maximal relative levels at about day 18. At day 20 and in newborn pancreases its synthesis is diminished relative to amylase and chymotrypsinogen (data not shown); in adult secretions it is a comparatively minor protein. The embryonic form shows a high degree of charge heterogeneity, which decreases in the adult form. Treatment of the proteins released by 18-d pancreases with a mixture of glycosidases from *Diplococcus pneumoniae* (containing neuraminidase, β-galactosidase, hexosaminidase, and endoglycosidase D activity[1]) reduces the heterogeneity of this component, similar to the reduction in heterogeneity seen in adult secretions. *D. pneumoniae* glycosidase treatment has no detectable effect on the other secretory proteins. Thus, glycosylation accounts for most of the heterogeneity of component *2* but does not account for the heterogeneity seen in other secreted proteins (e.g., protein *8*). Comparison of the heterogeneity of component *2* secreted by embryos of different ages (Fig. 1 *b–e*) and in the adult (Fig. 1*f*) suggests that its pattern of glycosylation changes during pancreatic development. Because protein *2* can be precipitated with glycogen and binds to Sepharose (a substrate analog), it seemed possible that it is an amylase. However, it is not precipitated with antiamylase antibody, and we have been unable to detect amylase activity associated with this component (data not shown).

We have compared proteins secreted from pancreases to those present in zymogen granules. 25 18-d pancreases and 30 adult pancreatic lobules were labeled with [^{3}H]leucine, and zymogen granules from each were prepared by the method of Meldolesi et al. (15). The isolated granules were then lysed, the membranes were removed by centrifugation at 100,000 *g* for 60 min, and the supernatant solutions were analyzed by the two-dimensional gel system. Autoradiographs of the labeled 18-d zymogen granule lysate show only proteins *1* (amylase), *2*, *4*, and *8* (chymotrypsinogen), the secretory subset (Fig. 3 *b*). Similarly, the adult zymogen granule proteins resemble the proteins secreted by the adult pancreas (Fig. 3 *a*). There are several notable exceptions such as proteins *5* and *6* and the basic proteins *10* and *11* in the zymogen granule lysate. The loss of the basic proteins may be the result of preferential binding to anionic (membrane) components during zymogen granule isolation and lysis.

The group of proteins released by the embryonic pancreas (arrows, Fig. 1 *a*) are detected throughout embryonic development but are absent from adult secretions (Fig. 1*f*). These proteins are also not detected in zymogen granules (cf. Fig. 1 *e* with Fig. 3 *a* and *b*). Several of these proteins released by early embryonic pancreases are absent or released at lower levels by day 18. Most of these proteins are also released by several of the other embryonic organs examined. Fig. 4 shows the proteins released by 16-d gut, lung, and liver and by mesenchymal tissue dissected from 14-d pancreas. (At this stage, the epithelial and mesenchymal components of the pancreas are not integrated, and can be easily separated.) The pancreatic proteins found in zymogen granules and in adult secretions (the secretory subset, proteins *1*, *2*, *4*, and *8*) are not released by these other tissues, consistent with the demonstration that this group consists of pancreatic digestive (pro)enzymes. Most of the other proteins released by the embryonic pancreas are also released by the gut, lung, and pancreatic mesenchyme. In contrast, the 16-d liver releases none of the proteins common to the other embryonic tissues; it releases only a limited set of specific proteins that may include albumin (68,000 mol wt) and transferrin (68,000 mol wt) (3). Because the liver demonstrates differentiated functions earlier than the other organs studied (24), 16-d liver may already be past the stage at which the released subset of proteins are detectable. The function of this class of released proteins is unknown.

Total Proteins Synthesized by the Embryonic Pancreas

Fig. 5 shows the two-dimensional gel profiles of total proteins synthesized in 14- through 18-d embryonic and adult pancreases during a 6-h incubation with [^{3}H]leucine. The overwhelming commitment of the adult tissue to the synthesis of digestive (pro)enzymes is illustrated by the similarity of the profiles of the total proteins synthesized (Fig. 5*f*) and the proteins secreted (Fig. 1*f*). It is estimated that >90% of total adult protein synthesis is dedicated to the synthesis of secretory proteins; only a few nonsecretory proteins are detected. However, if the same gel shown in Fig. 5*f* is exposed to the film 20 × longer, many other proteins appear (not shown); thus numerous other proteins are synthesized at very low levels. In contrast, in the embryonic pancreas the synthesis of large numbers of proteins (>200 species) is detectable at short exposure times. The patterns of the nonsecretory proteins, both in terms of the number and the relative intensity of spots, change very little from 14 to 18 d gestation. The few changes detectable are indicated by arrows in Fig. 5. The synthesis of secretory proteins, however, gradually increases until it becomes the dominant synthetic activity (20 d gestation and thereafter). A major quantitative and qualitative change in protein synthesis occurs between the late embryonic and the adult stage. A reduction in synthesis of nonsecretory proteins is accompanied by an increase in the synthesis of the specific secretory protein repertoire.

When pancreatic protein synthesis is examined immediately after dissection from the embryo, the synthesis of amylase and chymotrypsinogen is not detected until day 18 (Fig. 5). When the proteins secreted from 14 to 18 d gestation are analyzed (Fig. 1), chymotrypsinogen is detected as early as day 16, but amylase is not apparent until day 17. These results are inconsistent with earlier reports (14, 23, 26) showing large increases in the synthesis and accumulation of amylase and chymotrypsinogen beginning about day 15. It was found, however, that when embryonic pancreases are cultured in vitro the synthesis of amylase and chymotrypsinogen is detected earlier, about the same time predicted by the earlier studies. Fig. 6 shows expanded autoradiographs of the amylase, protein *2*, and chymotrypsinogen regions of the two-dimensional gels for 14-d pancreases and 14-d pancreases cultured for 1, 2, or 4 d. The synthesis of amylase and chymotrypsinogen can first be detected after 1 d in culture (equivalent to day 15) and protein *2* after 2 d in culture. Thus, there is a difference in protein synthesis in pancreases immediately after dissection and after culture for several days in vitro. This difference is primarily in

[1] Personal communication from Dr. William J. Grimes, Department of Biochemistry, University of Arizona.

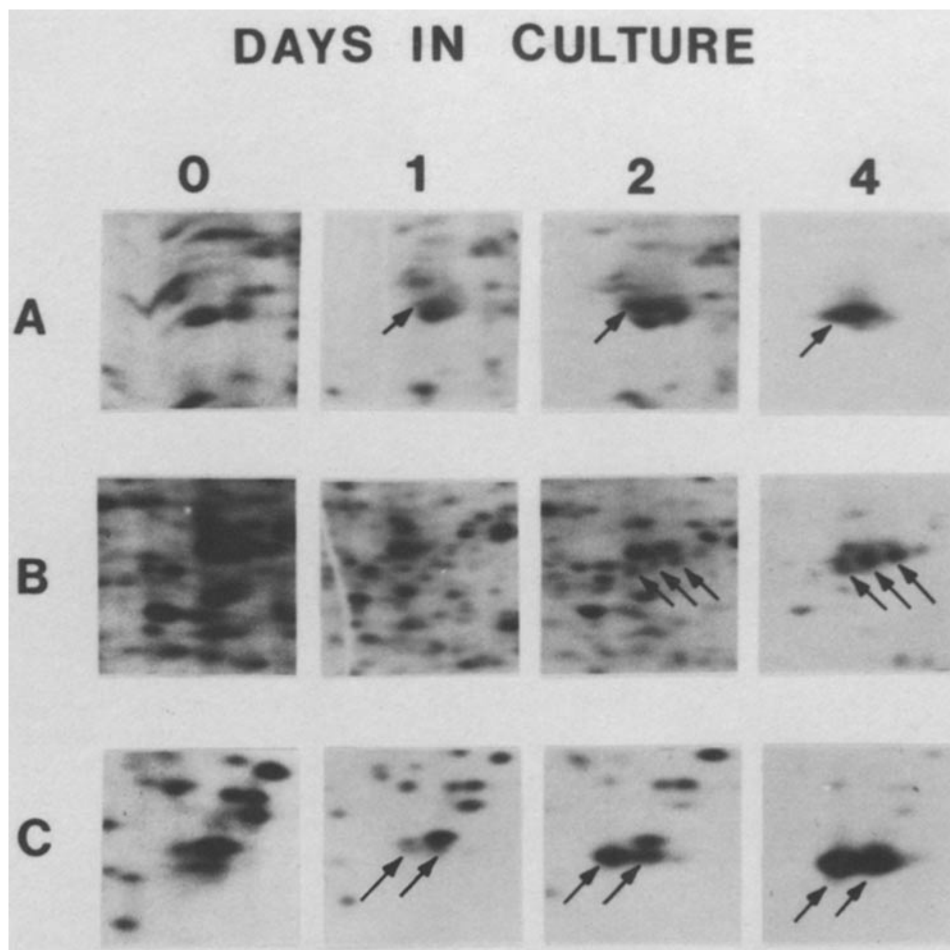

FIGURE 6 Detection of amylase, protein *2*, and chymotrypsinogen in cultured embryonic pancreases. Total proteins of 14-d pancreas, 14-d pancreases grown for 1 (14 + 1), 2 (14 + 2), and 4 (14 + 4) d in culture were labeled and run on two-dimensional gel electrophoresis as in Fig. 1. The autoradiograph region containing (*a*) amylase, (*b*) protein *2*, and (*c*) chymotrypsinogen are shown for each day. Arrows indicate the specific protein of each region (amylase, protein *2*, or chymotrypsinogen).

the synthesis of the pancreatic secretory proteins, as the synthesis of most other proteins appears similar in both cases.

Total Proteins Synthesized by Other Embryonic Tissues

To determine the limits of the pancreatic differentiative program, we have examined the repertoire of proteins synthesized in a number of embryonic cell types including gut, lung, liver, and mesenchyme. The two-dimensional patterns of the proteins synthesized by the 16-d gut and lung and the 14-d mesenchyme (Fig. 7 *a*, *b*, and *d*) are strikingly similar to the protein patterns of the embryonic pancreas (Fig. 5). In contrast, at 16 d gestation in the liver there is already substantial synthesis of what appear to be specific proteins (e.g., albumin and transferrin (3), and globins, as the early liver is known to be hemopoietic) (Fig. 4 *c*). This is consistent with previous reports showing early synthesis of differentiative proteins in the liver (24) and our demonstration of the specific secretion of some of these proteins (Fig. 4).

The similarity of the protein synthetic pattern of the pancreas, gut, lung, and mesenchyme is obvious from the remarkable constancy in both the number and relative intensity of spots detected when equal amounts of labeled material (200,000 cpm) from each tissue are applied to the gels. To illustrate these similarities, we photographically enlarged autoradiographs of Fig. 4 *a* (pancreas) and Fig. 7 *a* (gut), *b* (lung), and *c* (mesenchyme) and traced the major protein spots of a central region (enclosed within the borders, Fig. 7 *a*) so that direct comparisons could be made among the various tissues. In the comparison of gut and pancreas (Fig. 8 *a*), 82% of the prominent gut proteins are shared with the pancreas and, conversely, 95% of the prominent pancreatic proteins are shared with the gut (of 158 total proteins, 124 are common, 27 are only in gut, and 7 are only in pancreas). Similarly, in the lung (Fig. 8 *b*) 83% of the prominent proteins are shared with the pancreas and 92% of the pancreatic proteins are shared with the lung (of 154 proteins, 122 are common, 22 are only in the lung, and 10 are only in the pancreas). In the 14-d pancreatic mesenchyme (Fig. 8 *c*), 98% of the proteins are shared with the pancreas and 92% of the pancreatic proteins are shared with mesenchyme (of 132 proteins, 118 are common, 3 are only in mesenchyme, and 11 are only in the pancreas). There are very few tissue specific proteins detectable in these tissues at this developmental stage. In the gel regions analyzed in Fig. 8, there are only six gut proteins, one lung protein, three mesenchymal proteins, and three pancreatic proteins not detected in the other tissues (arrows, Fig. 8 *a–c*). This qualitative comparison does not illustrate the similarity also seen in the relative intensity of the spots in these tissues. In the tissues examined, both the number

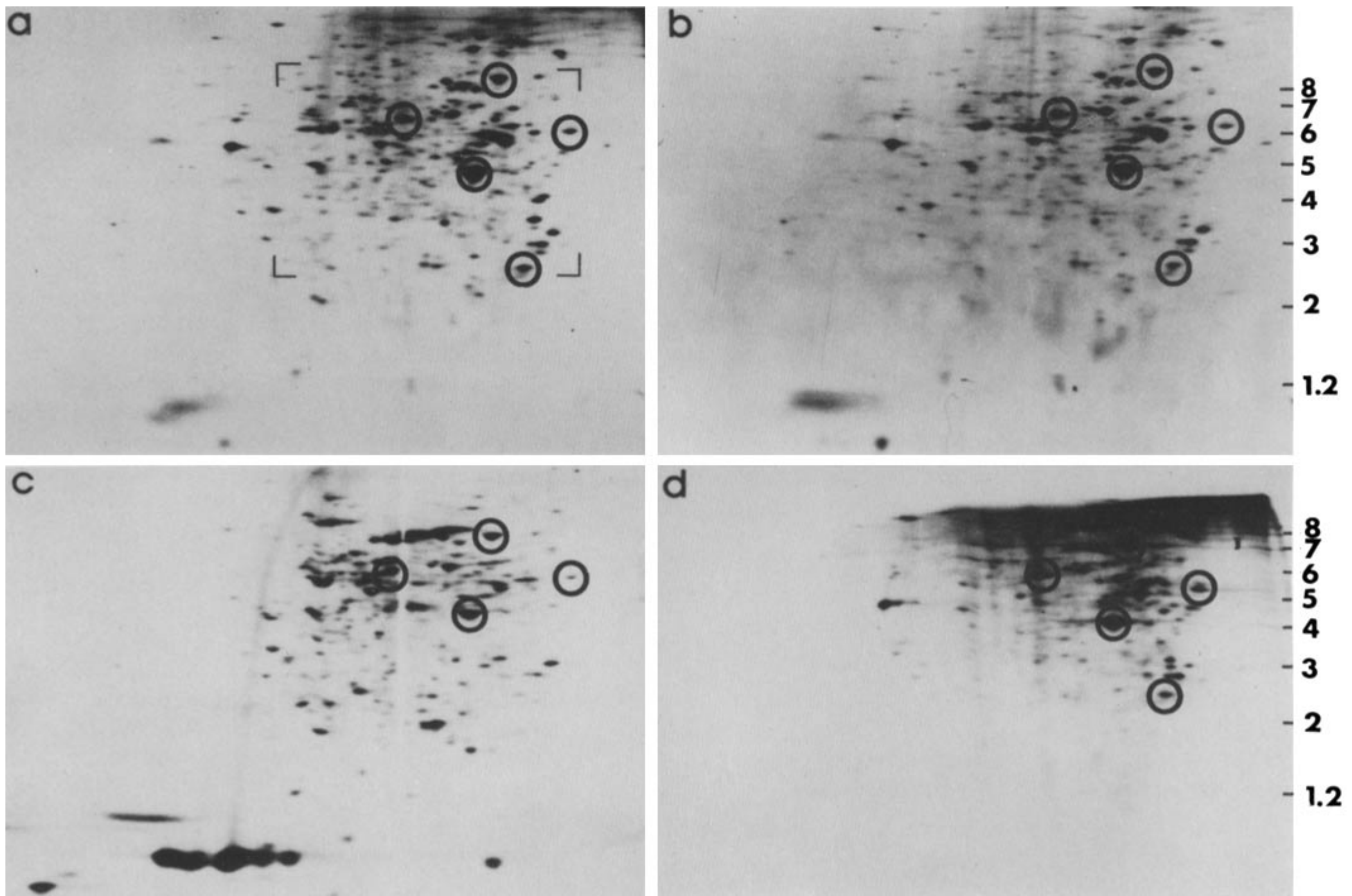

FIGURE 7 Two-dimensional gel electrophoresis of total proteins of embryonic gut, lung, liver, and mesenchymal tissue. Electrophoresis was performed as described in Fig. 1. *a*, 16-d gut proteins; *b*, 16-d lung; *c*, 16-d liver; *d*, 14-d pancreatic mesenchyme. Borders in *a* indicate region that is compared in Fig. 8. Circled spots are the same reference proteins circled in Fig. 5. The scale at the right indicates molecular weight $\times 10^{-4}$ as in Fig. 1.

of spots and their relative intensities indicate the similarity of the basic synthetic program for this set of detected proteins.

DISCUSSION

The two-dimensional gel electrophoretic analysis of proteins synthesized during pancreatic development is an important adjunct to our previous studies of the developing pancreas (5, 10, 11, 14, 20, 23, 26), because it allows independent analysis of specific molecular species as well as detection of a large fraction of the proteins synthesized by the tissue. The NEPHGE gel system detects proteins regardless of their isoelectric point (17). Between 200 and 300 pancreatic proteins are readily detected using this system; the sensitivity can be estimated by the detectability of amylase at early stages of development. Amylase is first detected in 14-d embryonic pancreases cultured 1 d in vitro (equivalent to 15 d gestation). Amylase synthesis at this time (calculated from earlier data [11, 20]) accounts for 0.1–0.3% of total protein synthesis. We estimate the procedure is $\sim 10\times$ more sensitive by measurement of spot intensity at different exposure times. Thus proteins accounting for $\geq$.01% of total proteins are detectable by this system. Whereas the great majority of proteins are detected, undetected proteins may play crucial regulatory roles in differentiation.

Many of the proteins secreted by the adult pancreas and present in zymogen granules were identified either by direct measurement of enzyme activity and/or by comparison with isoelectric points and molecular weights of known proteins (MacDonald and Rutter, unpublished observations; see also reference 27). The time at which the synthesis of these proteins is initially detected in embryonic development in general confirms our earlier results (14, 23, 26), with one significant exception. When the pancreases were dissected at 14 d gestation and cultured in vitro for various periods (the procedure followed in earlier experiments [14, 23, 26]), amylase and chymotrypsinogen synthesis was detected after 1 d in culture ($\cong$15 d gestation). However, these proteins were not detected until several days later when the pancreases were labeled directly after dissection. Therefore the synthesis of the cell-specific proteins is selectively affected by the culture conditions. Several of the (pro)enzymes, including trypsinogen, ribonuclease, and what appear to be lipase and proelastase, were not detected in embryonic pancreases until 20 d gestation. These proteins, however, were demonstrated by use of more sensitive techniques (enzyme assays and antibody precipitation [14, 23, 26]) to be synthesized several days earlier.

The present data emphasize the major changes in synthesis occurring during terminal differentiation. These changes occur relatively early for some genes (e.g., amylase and chymotrypsinogen) but are delayed for others (e.g., trypsinogen, ribonuclease, lipase, etc.). This pattern of early and late gene expression during the secondary transition was also detected in experiments of cell free translation of mRNA from embryonic pancreases (11). It is possible that different processes affect the

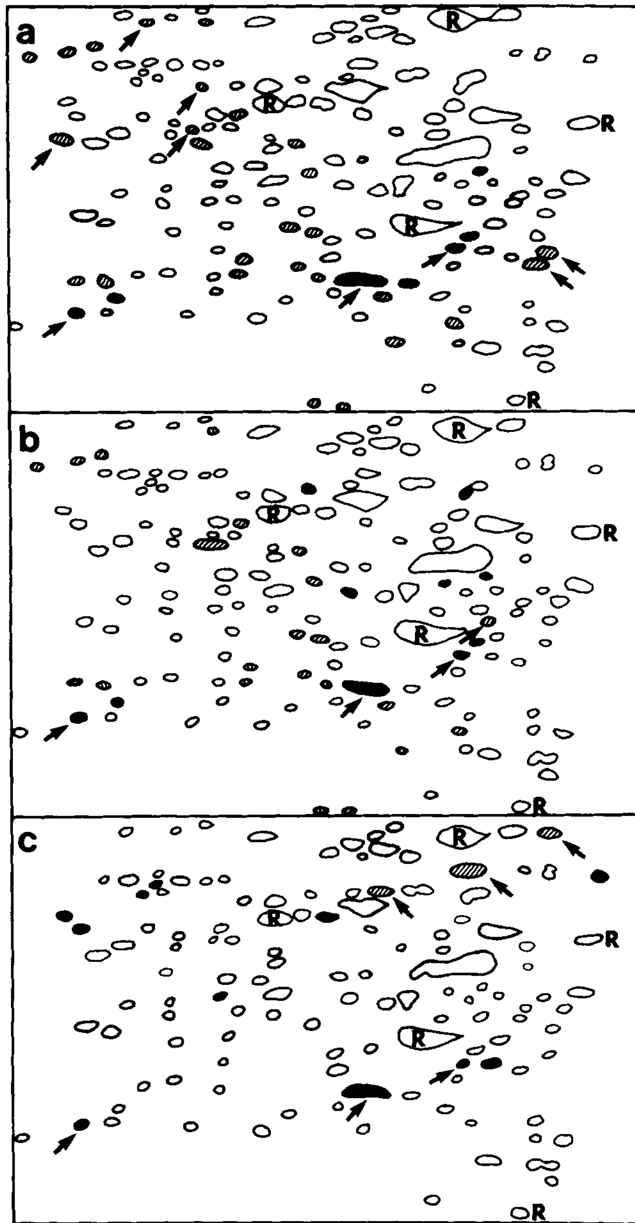

FIGURE 8 Detailed comparison of total proteins of the embryonic pancreas and total proteins of embryonic gut, lung, and mesenchyme. The central regions (indicated by borders in Fig. 7 *a*) of the total protein gels from 16-d pancreas, 16-d gut, 16-d lung, and 14-d mesenchyme were photographically enlarged and the major proteins of each region were traced. *a*, gut and pancreas proteins; *b*, lung and pancreas proteins; *c*, mesenchyme and pancreas proteins. Shaded spots indicate proteins detected in the pancreas but not in the other tissue with which it is being compared. Cross-hatched spots indicate proteins detected in the other tissue but not in the pancreas. Open spots indicate proteins common to the pancreas and the other tissues. Arrows indicate proteins specific to only one of the tissues analyzed. *R*, reference proteins circled in Figs. 5 and 7.

expression of the early and late sets of pancreas specific genes.

During the course of these studies we found that embryonic pancreases cultivated in vitro release a distinct subset of proteins into the culture medium. In the late embryonic and adult stages these are primarily found in zymogen granules and represent identified secretory proteins. The selectivity of the process in the early embryos is manifest, because the major species of the released proteins are not detected in gels of total cell proteins. Thus it is obvious that the accumulation of these proteins in the media is not the result of a random process such as cell lysis. The secretion of some pancreas-specific (pro)enzymes was detected as early as 15 d gestation; this secretion, however, is apparently insensitive to known secretagogues before 21 d gestation (6). The secretory mechanism itself is apparently operative at 15 d gestation, whereas the regulation of secretion develops later. In addition to the secretory proteins, another set of proteins accumulates in incubation media, especially in the early embryo. We do not know whether they are secreted or simply released from the cell surface; therefore, we have termed these the "released subset." This subset of proteins is not restricted to the pancreas, but is also produced by several other embryonic tissues (pancreatic mesenchyme, gut, and lung) in culture.

In contrast to the dramatic changes seen in the synthesis of the specific secretory proteins, the synthetic pattern of the total proteins of the pancreas was found to change very little during the crucial period of development. The numbers and relative intensity of spots are remarkably constant during the period of early development. Between the late embryonic stage and the adult, the synthesis of the majority of protein species gradually fades as the synthesis of the secretory proteins becomes dominant. At the adult stage >90% of the total protein synthesis in the pancreas is dedicated to the synthesis of the secretory digestive (pro)enzymes. Thus terminal differentiation of the acinar cells appears to involve the high-level expression of a limited number of genes (coding for digestive (pro)enzymes), whereas the pattern of expression of the remaining detected domain (coding for the majority of proteins) appears to change little. This pattern of differentiation was inferred earlier from different and incomplete evidence (24).

When the analysis of total protein synthesis was extended to several other embryonic tissues, a remarkable similarity in the synthetic pattern was found among pancreas, gut, and lung, (epithelial tissues) as well as mesenchyme (a mesodermal tissue). For example, at 16 d gestation, >90% of the proteins detected in the pancreas are also synthesized in these other tissues. The major exception (liver) is probably the result of the early differentiation of this tissue. Thus, the differentiation of several tissues seems to involve increased expression of a relatively small set of genes without changing markedly the expression of a set of proteins that appear common to several tissues. The proteins common to all tissues may be termed the constitutive set, those that are expressed in several but not all tissues, the semiconstitutive set, and those restricted to a specific tissue type, the differentiative set.

Besides the constitutive set of proteins and the cell-specific secretory proteins (some secretory proteins may be constitutive), there remains another set of proteins of undetermined magnitude, but of considerable probable significance. Some of these proteins may reside on the cell surface or in the chromosomal structure, and play crucial regulatory roles. These may include nonsecretory pancreas-specific proteins already detected or proteins that as yet remain undetected. For an understanding of the process of differentiation, these molecules, not the products of differentiation, must be identified and their functions delineated.

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