The Developmental Regulation of Amylolytic and Proteolytic Enzymes in the Embryonic Rat Pancreas

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SUMMARY

During the period of morphogenesis of the rat pancreas, the specific exocrine enzymes accumulation pattern from Day 13 through Day 14 to as late as Day 15 is a low and constant basal level of each specific protein (the protodifferentiated level). Subsequently, the levels of the exocrine enzymes increase dramatically. The specific activities of chymotrypsin(ogen), trypsin(ogen), (pro)carboxypeptidase A and B, and amylase increase approximately 1000-fold (differentiated stage). The inflection point of the typically sigmoid accumulation (plotted logarithmically) of the different enzymes occurs between 16½ and 18½ days. The initiation of these increases is not temporally coincident for all the proteins nor do the changes in specific activity of the enzymes appear to be coordinate. The levels of the enzymes in the adult pancreas average from several fold less to several fold more than those present in that of the newborn animal. Addition of agents implicated in altering the quantity of chymotrypsin and amylase in the adult animal have no effect on the in vitro embryonic accretion of these enzymes. Thus the levels appear to be independently modulated by unknown factors.

Determinations in embryonic and adult tissues other than pancreas indicate that (pro)carboxypeptidase A activity is present in significant quantity only in the pancreas. In all cases, specific activities are considerably less than that of the pancreatic protodifferentiated level. This substantiates the existence of a protodifferentiated stage in pancreatic development.

Enzyme assay of explants following organ culture of 13-day pancreatic rudiments yields a profile of specific protein accumulation coincident with that observed in vivo. Measurements of rates of biosynthesis of exocrine enzymes in vitro demonstrate a 30- to 60-fold increase during the developmental period studied (13 to 19 days). Inclusion of actinomycin D or 5-bromodeoxyuridine in vitro during the protodifferentiated period results in 90% inhibition of subsequent appearance of enzyme activity or biosynthesis (or both). Treatment at later times with these antimetabolites produces a progressively smaller effect. These results suggest that a DNA-linked process and, subsequently, RNA synthesis are required for expression of differentiated character. A multiphasic model of regulation in pancreatic organogenesis is discussed.

The pancreas is particularly well suited as a vehicle for the study of cytodifferentiation. The histology of both adult exocrine and endocrine regions is well defined. The exocrine tissue is arranged in an acinar configuration around a lumen, while the endocrine cells comprise the islets of Langerhans. The cytology is typical of secretory cells, the most prominent feature being the zymogen granules of the acinar cells and the secretory granules of the A and B cells. In addition, the secretory products of both exocrine (trypsinogen, chymotrypsinogen, RNase, amylase, etc.) and endocrine cell types ((pro)insulin, glucagon) have been extensively studied biochemically, and moderately sensitive and specific assays exist for most of them.

Several aspects of pancreatic development have been reported. The morphogenesis of the mouse and rat pancreas has been studied extensively (1–5). Furthermore, it has been shown (6–9) that pancreatic morphogenesis is a result of heterotypic interaction between epithelial and mesenchymal cells, as has been observed for a number of other tissues (cf. Ref. 7), and that normal histogenesis may proceed in vitro. Rutter et al. (8) reported the temporal pattern of accumulation of amylase and demonstrated that the appearance of enzyme activity was dependent on the presence of mesenchymal tissue or a factor derived from embryos rich in mesenchymal tissue (9). The present study extends these initial observations to another species and, moreover, examines the interrelationship of the accretion of several proteolytic and amylolytic enzymes. The observations presented here, especially when considered in the light of several other reports from this laboratory concerned with aspects of pancreas development (9–15), suggests plausible models for cytodifferentiation and its regulation in this and perhaps other tissues as well.
Enzymes—Bovine α-chymotrypsin (three times crystallized, salt free) and trypsin (either two times crystallized, salt free, or two times crystalized, 50% MgSO4) were purchased from Worthington. Crystalline bovine carboxypeptidase A and B were the generous gift of Drs. K. Walsh and H. Neurath.

Trypsinogen kinase of Aspergillus oryzae (an extracellular proteolytic enzyme active at low pH EC 3.4.14.27) was purified by a modification of the procedures of Prahl (16) and Hofmann and Shaw (17). One gram of Taka-diastase powder (Parke-Davis) was extracted at 4°C for 24 hours with glass-distilled water and centrifuged at 12,000 × g for 15 min to remove insoluble material. The supernatant was dialyzed overnight versus 10−4 M HCl, centrifuged again at 12,000 × g, and concentrated to approximately 2 ml by vacuum dialysis. The concentrated extract was equilibrated by dialysis with 0.01 M sodium acetate, pH 4.5, and applied to a column (0.9 × 17 cm) of DEAE-cellulose (Schleicher and Schuell) previously equilibrated with the same buffer. The column was washed with five column volumes of starting buffer and a linear gradient (0.0 to 0.4 M) of (NH4)2SO4 applied. Several protein species were resolved; the active fractions were pooled and used to activate trypsinogen (vide infra).

Substrates—N-Benzoyl-L-tyrosine ethyl ester and ε-amino-caproic acid were purchased from Mann Research Laboratories, Inc. and Nαtosyl-L-arginine methyl ester from Sigma Chemical Company, and used without further purification. Hippuryl-d-phenylalanyl-L-arginine, hippuryl-L-phenylalanine, and Nα-hippuryl-L-arginine methyl ester, all purchased from Sigma Chemical Company, O-Hippuryl-L-arginic acid was synthesized from L-arginic acid (α-hydroxy-4-quinodino-N-valeric acid) prepared by the method of Hamilton and Ortiz (18), and hippuryl chloride (19). L-Arginic acid (4.75 g, 0.027 mole) is triturated with 60 ml of dimethylformamide until most is dissolved, and 5.12 g (0.027 mole) of pyridine hydrochloride is rapidly added with mixing. To this suspension 5.63 g (0.28 mole) of hippuryl chloride is added followed by 2.51 ml (0.031 mole) of pyridine in 0.5 ml aliquots over a 10-min period. The flask should be protected from water vapor with a calcium chloride drying tube during these operations. Mixing is continued for 1 hour, and the flask is then stoppered and allowed to stand overnight.

Solvent is removed by rotary evaporation at 40°C, and the residual syrup is extracted several times with ethyl acetate until little or no yellow color is removed. The residue is then extracted several times with chloroform and traces of solvents removed in vacuo. The residue is taken up in approximately 20 ml of warm water (35-50°C), acidified with 1 N HCl to approximately pH 1, and left at 4°C overnight. The pale yellow crystals are collected on a filter, washed with ice-cold absolute ethanol, and dried in vacuo. Recrystallization is effected from warm water acidified as before and allowed to stand at 4°C and allowed to stand at 4°C followed by washing with ice cold ethanol and drying in vacuo. The white crystalline product gives a single Sakaguchi-positive spot (Rf = 0.71) when chromatographed on a thin layer of cellulose in butanol-pyridine-acetic acid-water (50:20:6:34) and is rapidly saponified in 0.1 N NaOH, yielding a Sakaguchi-positive spot corresponding to arginine (Rf = 0.49) and an ultraviolet-detectable spot near the solvent front corresponding to hippuric acid. Yield, 2.48 g (24.6%); m.p., 186-188°C with decomposition.

C16H13NO4·HCl
Found: C 48.17, H 6.17, N 6.44.

0-Hippuryl-L-arginic acid has been prepared previously and utilized as substrate for carboxypeptidase B (20, 21); however, the purification (repeated recrystallization of an amorphous glass) was laborious and the yields obtained were low (<10%).

Assays—Proteolytic enzyme determinations were based on existing spectrophotometric methods for chymotrypsin and trypsin (22), carboxypeptidase A (23, 24), and carboxypeptidase B (25). Measurements were made with a Gilmour model 2000 recording spectrophotometer maintained at a constant temperature of 25°C and with a full scale deflection of 0.1 absorbance unit. All assays were carried out in 1-cm cuvettes in a final volume of either 1.0 ml or 0.2 ml depending on the expected activity of the sample, with substrate concentrations and buffers as indicated for each enzyme: chymotrypsin, 4.5 × 10−5 M N-benzoyl-L-tyrosine ethyl ester in 0.05 M Tris-Cl, 0.05 M CaCl2; 25.6% (w/w) methanol, pH 7.65, at 4°C, 1.0 ml of 1 × 10−3 M NaCl, pH 7.65, at 4°C, 0.01 M CaCl2, pH 8.0; carboxypeptidase A, 5.0 × 10−4 M hippuryl-L-phenylalanine or 5.0 × 10−4 M hippuryl-d-phenylalanyl-L-arginic acid and 10−4 M ε-amino-caproic acid in 0.025 M Tris-Cl, 0.5 M NaCl, pH 7.65; carboxypeptidase B, 5.0 × 10−4 M Nα-hippuryl-L-arginine or O-hippuryl-L-arginic acid in 0.025 M Tris-Cl, 0.1 M NaCl, pH 7.65. In actual practice, in each case 0.9 ml or 0.18 ml of a substrate solution (1.1 times the final concentration of substrate indicated above) and an appropriate volume of buffer were placed in the cuvette, such that when the sample was added to initiate the reaction the final volume would be 1.0 ml or 0.2 ml. In this manner various sample volumes up to 0.1 or 0.20 ml, respectively, could be accommodated.

Winterberger et al. (26) have observed that bovine carboxypeptidase B possesses significant activity toward the substrates employed for assay of carboxypeptidase A. For this reason ε-amino-caproic acid has been included in all carboxypeptidase A assay mixtures at a concentration (10−3 M) sufficient to inhibit ≥95% of the carboxypeptidase B activity toward these substrates. It has subsequently been demonstrated that the activity of highly purified rat carboxypeptidase B hydrolyzes this substrate at a rate equal to that of bovine carboxypeptidase A (25); thus the specificity of the assay is assured.

All results were corrected for substrate blank rates and, in the case of chymotrypsin, for the contribution of the trypsin used inzymogen activation to the total rate. Units are expressed as micromole of substrate hydrolyzed per min for each enzyme and substrate; product and substrate ε values were either taken from the literature (22, 24, 26) or in the case of O hippuryl L arginine, an observed Δε of 585 liter mole−1 cm1 was determined by measurement of the change in absorbance after complete enzymatic hydrolysis of known concentrations of O-hippuryl-L-arginic acid. In cases where more than one substrate has been utilized for the assay of an individual enzyme (carboxypeptidase A and B), all results have been normalized to a single substrate with the use of conversion factors determined with activated rat pancreatic extracts (vide infra).

Amylase was assayed by micromodifications of both the methods of Van Loon et al. (27) and Bernfeld (28), which measure disappearance of the starch-iodine chromatophore and liberation of reducing sugar residues, respectively. In the case of the former procedure, 100 μl of 0.5 M Tris-Cl, 0.02 M NaCl, pH 7.2, and 100 μl of an 0.05% solution of soluble starch in the same buffer are pipetted into 100 μl disposable plastic test tubes (Falcon, Cock-\(\text{man}\)), mixed, and 20 μl of sample added with mixing to initiate the reaction. After 10 min incubation at room temperature, the reaction is stopped by the addition of 20 μl of a solution consisting of 1 part of 0.05 M Tris-Cl, 0.02 M NaCl, pH 7.2, and 1 part of a 1.5% KI-0.15% I2 solution in water. The absorbance at 540 nm is read within 10 min versus a blank containing no starch, together with a standard in which enzyme is absent, which should yield approximately 0.5 A540. Units are expressed as A540 per min. The substrate solution is a dilution in 0.05 M Tris-Cl, 0.02 M NaCl, pH 7.2, of a 0.1% stock starch solution obtained by boiling 100 mg of soluble reagent grade starch in 100 ml of the same buffer and storing at 4°C.

7. Calculated parameters of the Bernfeld (28) technique were modified to maximize sensitivity. Assays were carried out in 0.05 M histidine-Cl, pH 6.5 (instead of 0.02 M phosphate, 0.007 M NaCl, pH 6.9, used formerly) at 37°C in a volume of 50 μl (20-μl sample + 25-μl starch substrate) for time periods of 3 to 30 min and terminated by the addition of 50 μl of dinitrosalicylate reagent. After heating at 100°C for 5 min and cooling, 0.5 ml of water was added and the solution boiled for 300 min resulting in a substrate blank. Units are expressed as mg of maltose hydrolyzed released per min determined from maltose standards treated in the same manner.

When tested with commercial enzyme preparations (or dilutions of pancreatic extracts), the assays described yield a linear dependence on enzyme concentration, extending over several orders of magnitude in the case of the proteolytic enzymes and a 10-fold spread in the case of carboxypeptidase B.

1 T. G. Sanders and W. J. Rutter, unpublished results.
range with a maximum sensitivity of about 0.005 \( \mu g \) in the case of amylase. With the exception of carboxypeptidase A, maximum sensitivity was achieved in the protease determinations at low pH (33). Spectrophotometric measurements employing hippuryl-\(L\)-phenylalanine as substrate yielded a sensitivity in the same range as that indicated above for the other proteolytic enzymes. Low pH obviates the problem associated with subtraction of trypsin blanks when the latter enzyme is employed for autocatalytic activation of trypsinogen.

Organ Culture—Organotypic cultivation of embryonic pancreas utilizing procedures adapted from the previously described techniques of Oden (36), Fell (37), and Golosow and Grobstein (6). Pancreatic rudiments (12 or 13 days in \textit{utero}) were removed as indicated above, but under sterile conditions, and transferred to the surface of a Millipore filter (type THWP, Millipore Filter Corp.) supported at the air-medium interface in a disposable plastic organ culture dish (Falcon). Each dish accommodated four rudiments and contained 1 ml of medium composed of 90\% Eagle's basal medium (38) with Earle's salts (32), 10\% whole chick embryo extract, and an antibiotic-antimycotic mixture consisting of 100 units of penicillin, 100 \( \mu g \) of streptomycin, and 25 \( \mu g \) of fungizone per ml. Embryo extract was prepared essentially according to Cahn et al. (39) with the use of Earle's salts, but with the omission of hyaluronidase treatment, and stored at \(-20^\circ\). Other media components were purchased from Grand Island Biological Co. Cultures were maintained at 37\(^\circ\) in 5\% CO\(_2\)-saturated water vapor, with changes of medium every 48 hours, and harvested by passing the entire filter mount through a wash in Earle's balanced salt solution and transferring the tissue to a polyethylene micro test tube which was then stored at \(-20^\circ\).

Modification of the above procedure for specialized purposes included the following: in order to determine rates of synthesis of specific proteins, 8 hours before harvesting cultures as indicated above, the culture medium was replaced by a medium (Eagle's minimal essential medium (40) with Earle's salts (32) without leucine and unsupplemented with embryo extract, but containing antibiotics, as above) containing 200 \( \mu g \) per ml of dl-leucine, 5 \( \mu g \) per ml of asparagine (1:1 mixture), 5 \( \mu g \) per ml of L-phenylalanine. In these and subsequent experiments the assays are of enzyme activity; however, little if any activity is present without previous zymogen activation (see under "Materials and Methods"). Therefore, it is presumed that the activities measured represent the appearance of amylase, chymotrypsinogen, and trypsinogen, and (pro)carboxypeptidase A and B activities during in vitro pancreatic development is depicted in Figs. 1 to 3, respectively. In these and subsequent experiments the assays are of enzyme activity; however, little if any activity is present without previous zymogen activation (see under "Materials and Methods").

RESULTS

In Vivo Accumulation of Enzyme Activities—The course of appearance of amylase, chymotrypsinogen and trypsinogen, and (pro)carboxypeptidase A and B activities during in vitro pancreatic development is depicted in Figs. 1 to 3, respectively. In these and subsequent experiments the assays are of enzyme activity; however, little if any activity is present without previous zymogen activation (see under "Materials and Methods"). Therefore, it is presumed that the activities measured represent zymogen content. It is apparent that the same general sigmoidal pattern of accumulation is common to all the enzymes (with the exception of trypsinogen for which the data are incomplete, due to insufficient assay sensitivity); an early plateau period of low specific activity is followed by a substantial increase of several orders of magnitude to the relatively constant level found in the later embryo (20 to 21 days) and newborn pancreas. The relative proportions of the various proteins are subsequently altered somewhat in the adult state; for example, note the 5- to 8-fold reduction in the specific activities of chymotrypsinogen (Fig. 2) and (pro)carboxypeptidase A (Fig. 3) between newborn and adult stages. The data obtained for amylase describe the same curve irrespective of the assay method employed. When the same data are considered on a linear ordinate (rather than with a log scale as shown here), the majority of enzyme accumulation occurs during the later stages (17 to 20 days) of the transition.
parallel.

ally superimposable, and the alteration in chymotrypsin(ogen)
and (pro)carboxypeptidase A activities after birth occurs in
that the regulation of the synthesis of all the enzymes is not
exception of trypsin(ogen) to (pro)carboxypeptidase B, the ratios
carboxypeptidase A to (pro)carboxypeptidase B vary between
2.1 to 164 and 0.29 to 7.07, respectively. Similarly, with the
that the ratios of amylase to (pro)carboxypeptidase I and (pro)-
proteins are regulated coordinately. In fact, the developmental
ordinate since there is no turnover of these proteins (12) ; how-
ever, the data do not preclude the possibility that some of the
above, that the changes in enzyme content do not occur in con-
trypsin(ogen), as well as (pro)carboxypeptidase A and (pro)car-
supra) indicates, in addition to the temporal differences noted
A and (pro)carboxypeptidase B, respectively. Similarly, with the
increase in enzyme content during embryonic life, when expressed
as molecules per pancreatic cell, i.e. normalized to DNA rather than protein content (15). In addi-
tion to the indication of a lack of constant proportionality as dis-
cussed above, the results demonstrate that the molecular concen-
trations present in the early rudiments (13 to 15 days) are
significant (approximately 10^4 molecules per cell), suggesting a
phase in the differentiation of the exocrine cells, designated the
protodifferentiated state. In addition, the magnitude of the
increase in enzyme content during embryonic life, when expressed
as molecules per cell (normalized to DNA), is approximately
8-fold greater than that observed as specific activity (normalized
to protein) due to a concomitant increase in total exocrine cell
protein.

**Analysis of (Pro)carboxypeptidase A Activities in Other Tis-
sees**—In order to examine the hypothesis that the exocrine
proteins were cell-specific, evidence was sought to confirm the fact
that the level of enzyme activity present in the protodifferenti-
ated pancreas did not represent a gratuitous synthesis present
in all cells of the animal. To this end, assays of (pro)carboxy-
peptidase A activity present in other tissues at similar develop-
mental times were performed (using hippuryl-L-phenyllactic
acid as substrate for maximal sensitivity, see under "Materials
and Methods"). The results, presented in Table II, are calcu-
ated in a manner similar to the data in Table I (15) and indicate
that in most of the tissues examined, activities (molecules per
cell) are 1 to 2% that of the protodifferentiated pancreas. In
the several cases where significant rates were observed, each
extract was subjected to reactivity in the presence of 10^{-4} M
1,10-phenanthroline after preincubation with the inhibitor (10^{-4} M)
for 30 min at 0° (a procedure shown to destroy <90% of the
carboxypeptidase A activity present in rat pancreatic extract)
Table I
Concentrations of specific exocrine proteins during pancreatic development

Data of Figs. 1 to 3 (specific activity normalized to protein) recalculated as molecules per cell (normalized to DNA) assuming 7.12 x 10^-14 g of DNA per cell (92). The rate constants employed were those of highly purified rat enzymes in the case of amylase (47), trypsin (93), chymotrypsin, and carboxypeptidase B (75.6 and 78.7 units per mg, respectively) or the bovine homolog for carboxypeptidase A (35). Molecular weight values utilized were either taken from the literature (47, 48, 93) or determined for rat enzymes or zymogens (or both) in this laboratory.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>13 Days (protodifferentiated)</th>
<th>15 Days</th>
<th>17 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Molecules per cell</td>
<td>Molecular ratio</td>
</tr>
<tr>
<td>Amylase</td>
<td>0.0132</td>
<td>4.2 x 10^4</td>
<td>2.10</td>
</tr>
<tr>
<td>Chymotrypsin (ogen)</td>
<td>0.00655</td>
<td>1.3 x 10^4</td>
<td>6.50</td>
</tr>
<tr>
<td>(Pro)carboxypeptidase A</td>
<td>0.000833</td>
<td>1.4 x 10^4</td>
<td>0.70</td>
</tr>
<tr>
<td>(Pro)carboxypeptidase B</td>
<td>0.00172</td>
<td>2.0 x 10^4</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table II
Analysis of (pro)carboxypeptidase A activity in other tissues

Embryonic rudiments or adult fragments of the tissue indicated were excised and assayed for (pro)carboxypeptidase A with the use of hippuryl-naphthylactic acid as substrate as described under "Materials and Methods." Samples in which significant activity was observed were reassayed in the presence of 1,10-phenanthroline after preincubation with the inhibitor (10^-5 M) for 30 min at 0°C. Molecules of (pro)carboxypeptidase A calculated as indicated in Table I.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific activity</th>
<th>Molecules per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Newborn</td>
<td>Adult</td>
</tr>
<tr>
<td></td>
<td>14 Days</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>2.1 x 10^-4</td>
<td>1.8 x 10^4</td>
</tr>
<tr>
<td>Liver</td>
<td>1.8 (1.9) x 10^-4</td>
<td>1.3 (1.2) x 10^-3</td>
</tr>
<tr>
<td>Spleen</td>
<td>6.5 x 10^-4</td>
<td>&lt;2.9 x 10^-4</td>
</tr>
<tr>
<td>Parotid</td>
<td>&lt;2.9 x 10^-4</td>
<td>&lt;2.9 x 10^-4</td>
</tr>
<tr>
<td>Submaxillary</td>
<td>1.4 (1.5) x 10^-4</td>
<td>2.9 x 10^-4</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.0 (2.8) x 10^-3</td>
<td>1.3 (1.2) x 10^-3</td>
</tr>
<tr>
<td>Muscle</td>
<td>5.3 (6.9) x 10^-4</td>
<td>&lt;2.9 x 10^-4</td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;2.9 x 10^-3</td>
<td>3.6 x 10^-4</td>
</tr>
<tr>
<td>Lung</td>
<td>5.9 x 10^-3</td>
<td>1.2 x 10^-3</td>
</tr>
</tbody>
</table>

* Micromoles of hippuryl-naphthylactic acid per min per mg of protein; values in parentheses indicate treatment with 1,10-phenanthroline.

Due to the zinc metalloenzyme nature of the protein (45, 46). In no case did a non-pancreatic tissue extract show inhibition under these conditions as indicated by the specific activities shown in parentheses (Table II, Column 2 to 4); thus only 10% of this observed rate, at most, may be attributed to conditional (pro)carboxypeptidase A, and in these instances this corrected value is expressed as molecules per cell (Columns 5 to 7). (The activity remaining presumably reflects a relatively nonspecific esterase present in these organs.) The data of Table II were obtained with activated tissue extracts; similar results occurred in the absence of potential zymogen activation.

These observations, therefore, suggest that the genes involved in the production of the pancreatic proteins are expressed selectively in this tissue and, furthermore, that the protodifferentiated levels of activities observed represent a stage in the differentiation of this organ.

In Vitro Analysis of Enzyme Accumulation—The further analysis of the process of pancreatic differentiation has utilized in vitro organotypic culture procedures (see under "Materials and Methods"). Although the rate of growth (i.e. cell replication) observed in vitro is substantially less than occurs in vivo, the accumulation of the individual enzymes (expressed as specific activity) is temporally coincident with that observed in vivo as shown in Fig. 4 to 6. The curves indicated for amylase, chymo-
Effect of RNA and DNA Antimetabolites—The effects of the inhibitors actinomycin D and 5-bromodeoxyuridine on both enzyme accumulation and biosynthesis were then tested in vitro. Each inhibitor was included in the culture medium for 48-hour periods as indicated in Table III and the specific activity of amylase and chymotrypsin(o gén) determined after 6 days in culture. The results (Table III) demonstrate that when applied during the protodifferentiated period, each inhibitor caused essentially total cessation of enzyme accumulation (<10% of normal control). However, as the time of treatment with antimetabolites is shifted progressively later in the transition period, the accretion of enzyme activity becomes insensitive to the presence of an inhibitor. The data for amylase are presented graphically in Fig. 7 as percentage of inhibition of enzyme accumulation (specific activity) together with a linear representation of normal amylase accretion and biosynthesis.

Fig. 8 records the results of a similar experiment in which the effect of 5-bromodeoxyuridine and actinomycin D on chymotrypsin(o gén) biosynthesis (in contrast to enzyme activity in the case of amylase, above (Fig. 7)) was determined after 4 and 6 days in vitro, respectively. A more extensive analysis of the effects of 5-bromodeoxyuridine has now been completed and is reported elsewhere (94).\(^8\)

\(^8\) S. Githens, III, R. L. Pictet, and W. J. Rutter, in preparation.

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Embryonic age (days)}\)

\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Days in culture}\)

\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Embryonic age (days)}\)

\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Embryonic age (days)}\)

\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

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\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Embryonic age (days)}\)

\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Embryonic age (days)}\)

\(\text{Days in culture}\)

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\(\text{Days in culture}\)

\(\text{Amylase (mg moles/min/mg protein)}\)

\(\text{Days in culture}\)

\(\text{Embryonic age (days)}\)
FIG. 7 (left). Amylase biosynthesis and effects of actinomycin D and 5-bromodeoxyuridine on amylase accumulation. The accumulation of amylase (plotted linearly, ◦), rate of amylase biosynthesis (▲), effects of actinomycin D (10⁻⁸ M, ◦), and 5-bromodeoxyuridine (150 μg per ml, ▲) are taken from Fig. 1 and Table III, respectively. The effect of the antimetabolites is plotted as percentage of inhibition of the enzyme activity normally present at 19 days (6 days in vitro) versus the time at which treatment was initiated. Amylase biosynthesis determinations utilized 13-day pancreatic rudiments, cultured for the periods indicated and then subjected to labeling with [³H]leucine for 8 hours as described under “Materials and Methods.” After homogenization, the extracts were electrophoretically resolved in polyacrylamide gels, the gels fractionated, and the radioactivity in the region(s) corresponding to amylase determined. (Experimental details are presented by Kemp et al. (12).) NB and A indicate newborn and adult, respectively; BTEE denotes N-benzoyl-L-tyrosine; BUdR, 5-bromodeoxyuridine.

oppressive in this regulation was tested independently for its effect on the embryonic accumulation of the two enzymes in vitro by supplementing the culture medium as indicated under “Materials and Methods.”

The effects of alteration in either glucose or amino acid concentration of the medium on accretion of amylase and chymotrypsin(ogen) are shown in Table IV. Although differences in the specific activities of each enzyme are observed under the different culture conditions, the results are within the normal range of values (see Figs. 4 and 5). Furthermore, the variation does not appear to be selective; i.e. the changes in amylase to chymotrypsin(ogen) ratio are neither significant nor consistent with respect to effector or time. More extensive recent experiments have demonstrated an effect of increased amino acid concentration on the general vitality of the embryonic explant, and concomitantly, the specific activities of the exocrine proteins are also increased indiscriminately.²

Table V indicates the results obtained for the same two enzymes when the medium is either supplemented with insulin or glucagon, or additions of antisera to each hormone are made in an effort to remove the relevant endogenous activity and thus preclude its effect, if any, on the exocrine cells. The values for amylase and chymotrypsin(ogen) specific activities are again within the experimental errors inherent in the analysis.

DISCUSSION

The Protodifferentiated State

The results of determinations of pancreatic enzyme activity during development have yielded patterns of accumulation which suggest a model of cytodifferentiation for the exocrine regions of the organ previously presented elsewhere, consisting of three stages or phases and three transitions or regulatory events in the process of pancreatic function during the life of the organism (14). The protodifferentiated state was defined initially by the finding that significant and constant specific activities of exocrine proteins are present during early morphogenesis of the pancreatic rudiment (13 to 15 days), but are not detected in other organs (as demonstrated here with (pro)carboxypeptidase A). Additional experiments involving microassay of both lipase (10) and

² B. Walther, L. B. Rall, and W. J. Rutter, unpublished results.
Pancreatic rudiments (13 days) were excised and cultured either in normal medium or in the presence of actinomycin D (10^-6 M) or 5-bromodeoxyuridine (150 μg per ml) for 48-hour periods, as indicated, and the specific activity of amylase and chymotrypsin determined after 6 days in vitro. (See under "Materials and Methods" for details.)

**Table III**

<table>
<thead>
<tr>
<th>Culture period</th>
<th>Chymotrypsin(ogen)</th>
<th>Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 + 3 Days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (no additions)</td>
<td>0.1015</td>
<td>9.01</td>
</tr>
<tr>
<td>Glucagon, 5 μg per ml</td>
<td>0.1215</td>
<td>9.01</td>
</tr>
<tr>
<td>Anti-insulin serum, 1%</td>
<td>0.1285</td>
<td>0.20</td>
</tr>
<tr>
<td>Anti-glucagon serum, 1%</td>
<td>0.1285</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* Specific activity (units per mg of protein), as defined under "Materials and Methods.

**Table IV**

<table>
<thead>
<tr>
<th>Culture period</th>
<th>Chymotrypsin(ogen)</th>
<th>Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 + 3 Days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (no additions)</td>
<td>0.102</td>
<td>8.01</td>
</tr>
<tr>
<td>Glucose, five times</td>
<td>0.124</td>
<td>8.01</td>
</tr>
<tr>
<td>Amino acids, five times</td>
<td>0.122</td>
<td>13.46</td>
</tr>
</tbody>
</table>

* Specific activity (units per mg of protein), as defined under "Materials and Methods.

The existence of the protodifferentiated state as a transient period during which significant pancreatic exocrine enzyme activity (and thus gene expression) is present, implies that a previous regulatory event has occurred, resulting in a transition of predifferentiated cells (devoid of pancreas-specific proteins) to the protodifferentiated condition. Evidence that such a primary transition occurs, in fact, has been presented and discussed (2, 3, 10, 11, 14).

The main focus of the present experiments has been on the characterization of the substantial increases in exocrine pancreatic enzymes observed in vitro (resulting in the differentiated stage) and an attempt to elucidate the causal factors involved in the mechanism of this secondary regulatory transition using in vitro techniques. Although the changes in specific activity observed in Figs. 1 to 3 are of a generally similar nature, as discussed previously, the temporal asynchrony and noncoordinate aspects suggest that the regulation cannot be mediated in a direct manner entirely by a common causal agent, as, for example, either a rate limitation imposed by intracellular ribosome or rRNA concentrations (or both) or an operon system analogous to that of microorganisms (65). However, the possibility of an indirect regulation imposed via a general inductive effect on the exocrine cells cannot be eliminated; rather, such a general mecha-
nism, subject to secondary controls, is proposed. An alternative hypothesis, the selective synthesis of individual pancreatic enzymes by different subpopulations of exocrine cells, although possible, does not seem warranted since immunohistological analysis of specific protein tissue distribution has demonstrated an apparent functional equivalence of acinar cells (66-68).

The electron microscopic studies of developing pancreas of the mouse (1, 4, 5) and the extensive recent studies of the rat (2, 69) indicate that exocrine cell differentiation, both in vivo and in vitro, occurs primarily between 13 to 16 and 14 to 18 days of gestation, respectively, in the two species. These observations suggest a parallel sequence of biochemical and morphological events in which the formation of intracellular organelles associated with the synthesis, storage, and secretion of pancreatic exocrine enzymes is coordinated with the observed accumulation.

The observation that enzyme accumulation, in addition to histogenesis, follows a normal temporal course in vitro (Figs. 4 to 6) allowed an investigation of possible parameters operative in the secondary transition. The results of incorporation experiments (of which the relevant data for amylase and chromotropinogen are presented in Figs. 7 and 8, respectively) demonstrate that the rates of biosynthesis of the exocrine proteins are substantially elevated during the progress of in vitro pancreatic cytodifferentiation, and thus the increases observed in enzyme specific activity are directly attributable to de novo synthesis (see also Kemp et al. (12)).

The transient inhibitory effect of actinomycin D on the subsequent increases in enzyme specific activity and biosynthesis (Table III, Figs. 7 and 8) implicates the obligatory synthesis of a stable RNA species in the secondary transition (during the protodifferentiated state). An analogous change from sensitivity to resistance to actinomycin D has been recorded in a number of other developing systems: crystallin metabolism during fibrogenesis of vertebrate lens (70, 71), myogenesis (72), reticulocyte maturation, i.e. hemoglobin production (73, 74), and coococin synthesis and accumulation in the galea of the silkworm (60). It has been suggested (60) (on the basis of a quantitative consideration of rates of transcription and translation) that the production of a relatively stable specific mRNA may account for this transient pattern of actinomycin D inhibition and also the increase in tissue specific protein synthesis and resultant accumulation.

An analysis by Wessells and Wilt (75) of RNA species affected by in vitro actinomycin D treatment of embryonic mouse pancreas during the interval which results in an arrest of normal morphogenesis (8, 76, 77) demonstrated that the synthesis of ribosomal RNA was substantially inhibited. As similar effects were observed when inhibitor treatment was initiated later (at a time when cytodifferentiation is refractory to its presence), this lack of sensitivity is not due to the failure of actinomycin D to penetrate the cells. Thus, it seems likely that in order for subsequent organogenesis to occur, the synthesis of rRNA is also essential, but probably not sufficient, during the protodifferentiated state.

Wessells (77) and Walther et al. (94) observed that 5-bromodeoxyuridine and 5-fluorodeoxyuridine cause a block to cytodifferentiation. The specific effect of 5-bromodeoxyuridine on biochemical indicators of exocrine cell function, amylase, and chymotrypsinogen (ogen) (Table III, Fig. 7) suggest the existence of some DNA-linked process as a prerequisite to RNA synthesis. Walther et al. (94) have demonstrated a specific inhibitory effect of 5-bromodeoxyuridine on rates of biosynthesis of exocrine proteins. A similar response to 5-bromodeoxyuridine treatment occurs during myogenesis (78, 79), erythropoiesis (73), chondrogenesis (80, 81), etc.; see Rutter et al. (82) for a recent review and a discussion of possible modes of action of the inhibitor.

Modulations of Exocrine Levels in Differentiated Pancreas—The observation of Desmoule et al. that in the adult rat the specific activities and rates of biosynthesis of chymotrypsin and amylase were altered as a result of dietary or hormonal influences (tendency regulation or modulation) (49-51) prompted an investigation of the embryonic effects of these effectors in vitro. This was particularly pertinent since insulin accumulation in the embryonic endocrine pancreas slightly precedes the majority of the exocrine proteins (11), and glucagon concentrations comparable to that of the adult are found as early as 13 days gestation (84). No significant effect on the embryonic accumulation of amylase or chymotrypsin was observed with any of the potential effectors; thus, there is no evidence for the precocious induction of these exocrine proteins by either hormone, as has been observed in fetal liver for tyrosine aminotransferase and seirine dehydrogenase (85, 86), enzymes also known to be regulated in the adult by dietary and hormonal agents (87-89). This result was obtained even though (a) the concentration of glucose used approximates that of a diabetic animal (in which the most pronounced effects previously were seen in the adult case (50)); (b) treatment of adult rats (approximately 200 g) with the relatively specific diabetogenic agent streptozotocin (90) (2-deoxy-2-(3-methyl-3-nitrosoureido)-d-glucopyranose, 65 mg per kg of body weight, intravenously results in a 20-fold reduction in pancreatic amylase activity; and (c) the injection of anti-insulin serum causes clinical symptoms of diabetes (91). In the present case the antibody may not have penetrated the explant sufficiently in vitro to ensure complete removal of endogenous hormone activity from the environment of the exocrine tissue. Recent experiments from this laboratory have shown that glucocorticoids in physiological concentrations selectively enhance the accumulation of total exocrine proteins and alter the relative levels of specific protein in the developing rat pancreas. Kulka and collaborators (95, 96) have already demonstrated an effect of glucocorticoids on the developing chicken pancreas. Thus the maturation of the pancreas may be controlled by this hormone.4

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The Developmental Regulation of Amylolytic and Proteolytic Enzymes in the Embryonic Rat Pancreas
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