Published November 1, 1977

GLUCOCORTICOIDS MODULATE THE IN VITRO DEVELOPMENT OF THE EMBRYONIC RAT PANCREAS

LESLIE RALL, RAYMOND PICTET, SHERWOOD GITHENS, and WILLIAM J. RUTTER

From the Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143. Dr. Rall's present address is Los Alamos Scientific Laboratory, Los Alamos, New Mexico 87545, and Dr. Githens' present address is the Department of Biological Sciences, University of New Orleans, New Orleans, Louisiana 70122.

ABSTRACT

The effect of the glucocorticoid analogue, dexamethasone, on the development of the embryonic pancreas was studied in tissue culture. It specifically enhances the accumulation of exocrine enzymes without altering the level of general cell proteins. The enhancement, however, is not symmetrical: the cellular levels of the two major exocrine products, amylase and chymotrypsinogen, are increased about 10- and 2-fold, respectively. Two other zymogens that are present in minor quantities, procarboxypeptidases A and B, are also increased, whereas no effect is seen on lipase A. Coordinate with these effects on synthesis, there is a dramatic change in the morphology of dexamethasone-stimulated acinar cells. Their number of zymogen granules is higher and crystalline arrays are found in the rough endoplasmic reticulum. Dexamethasone also inhibits cell replication, perhaps by selectively inhibiting the last cell divisions of the culture period. At the same time, there is a disproportionate reduction in the insulin content of cultured rudiments.

We find that pancreatic development is normal in the absence of dexamethasone and that this glucocorticoid does not precociously induce the appearance of the specific secretory products, but rather enhances by a constant degree their synthesis and accumulation. Therefore, we conclude that glucocorticoids may play a modulatory but not an inductive role in pancreatic development.

KEY WORDS pancreas · in vitro development · glucocorticoids

Glucocorticoids play a developmental role in a variety of tissues, such as retina (16, 28, 36), skin (47), liver (10, 17, 32), lung (1), intestine (25, 26) and mammary gland (24, 48). In these tissues, glucocorticoids cause a precocious or enhanced accumulation of certain proteins and cellular organelles characteristic of the differentiated state. Where tested, these embryonic tissues contain glucocorticoid-specific receptors (1, 13, 15, 21).

We report here the effects of glucocorticoids on the in vitro development of the embryonic rat pancreas. This system has been extensively described at both the morphological and molecular levels (30, 31). As in vivo, the embryonic pancreas cultivated in vitro differentiates into exocrine (mostly acinar) and endocrine (largely A and B) cells. The acinar cells synthesize a set of digestive enzymes and the endocrine A and B cells produce the hormones glucagon and insulin, respectively.

We find that glucocorticoids (*a*) increase to different degrees the cellular levels of several exocrine enzymes (amylase in particular is induced to

Published November 1, 1977

supernormal concentrations), (*b*) "inhibit" cell division in the cultured rudiment, and (*c*) decrease disproportionately the gland content of insulin. Our studies of these effects suggest that glucocorticoids modulate but are not themselves the initiators of developmental events in the pancreas.

MATERIALS AND METHODS

Organ Culture and Tissue Preparation

Day-13 rat (Sprague-Dawley) embryonic pancreases were maintained in organ culture as previously described (19). The growth medium consisted of 90% Eagle's minimal medium supplemented to three times the usual level of the essential amino acids, 10% chick embryo extract (37), 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of fungizone. Stock solutions (10^{-3} M) of dexamethasone and hydrocortisone were dissolved in absolute ethanol and were stored at −20°C. Culture media with or without added steroids were prepared and changed daily. Control values were not changed by the presence of even 1% ethanol.

At specified times, cultured rudiments were harvested (15–20 explants/tube early in development and three to four explants/tube late in development) and stored in Spinco microfuge tubes (Beckman Instruments, Spinco Div., Palo Alto, Calif.) at −75°C. At the time of assay, they were thawed and subsequently sonicated in an aliquot (usually 150 μl) of sterile distilled water as detailed in reference 19.

Assays

The procedures for assaying insulin and the exocrine proteins in pancreatic sonicates have been described. The zymogens were first activated with trypsin and then assayed with the following substrates: *N*-benzoyl-L-tyrosine ethyl ester (BTEE) (chymotrypsin); hippuryl-DL-phenyllactic acid (HPLA) (carboxypeptidase A), and *O*-hippuryl-L-argininic acid (HAA) (carboxypeptidase B). Amylase activity was measured by a micromodification (42) of the Bernfeld technique (2) and lipase A was assayed colorimetrically using β-naphthyl stearate as a substrate and in the presence of 0.04 M sodium taurocholate (3).

Pancreatic specific enzyme activity is generally expressed in units, that is, micromoles of substrate hydrolyzed per minute. These units can be converted to milligrams of enzyme protein since the activities of the pure rat enzymes have been determined. For example, amylase assays depend upon the measurement of reducing sugar residues released during the course of starch hydrolysis. With pure enzyme and our asssay conditions, 260 mg of maltose are released per min per mg of amylase (42). Similarly, with the assays described by Sanders and Rutter (43), BTEE is hydrolyzed by pure activated chymotrypsinogen at the rate of 80 U/mg of enzyme and HAA is hydrolyzed by pure activated procarboxypeptidase B at the rate of 270 U/mg of zymogen (41). In the case of lipase A and procarboxypeptidase A, the turnover numbers of bovine enzymes (40) were used since these two rat enzymes have not been purified to homogeneity.

Insulin was assayed by a micromodification (6) of the double antibody technique (27) with rat insulin as a standard (33, 34). Therefore, in the assay procedure, identical molecules compete for the insulin antibody and the values are read directly as picograms of hormone. Protein determinations were made by a micromodification (39) of the Folin-Lowry assay. DNA and RNA contents were measured by the diphenylamine (5) and orcinol (9) procedures, respectively. The sensitivity of the RNA assay was increased by replacing ferric chloride with cupric chloride (20) and the values were corrected for the known concentrations of DNA in the samples.

Pancreatic tissues were prepared for light and electron microscopy as described previously (19).

Materials

All culture media solutions were from Grand Island Biological Co. (Grand Island, N. Y.), except embryo extract which was prepared in the laboratory. Starch was obtained from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.), BTEE from Schwarz/Mann (Orangeburg, N. Y.), HPLA from Cyclo Chemical Co. (Los Angeles, Calif.), and β-naphthyl stearate from Schwarz/Mann. HAA was synthesized by Dr. T. G. Sanders (41) and rat insulin was a gift from Dr. Schlicktkrull of Novoterapeutisk (Copenhagen, Denmark).

RESULTS

Glucocorticoids Increase the Protein to DNA Ratio in the Developing Rat Pancreas

The early embryonic rat pancreas explanted on day 13 continues to develop in vitro under our conditions. Over a 7-day culture period corresponding to the period between days 13 and 20 of embryonic development, the total DNA content of the explant increases six- to sevenfold and the total protein content 15-fold. Early in development (day 13), when the levels of the cell-specific products are low, the protein to DNA ratio is ~10 (see Fig. 1). At the end of *in utero* development (day 20), the cells are larger, enriched in rough endoplasmic reticulum and contain high levels of secretory proteins. These changes correlate with an increase in the protein to DNA ratio to 70. In vitro, the protein to DNA ratio increases only to 25. If dexamethasone (10^{-7} M) is included in the nutrient medium, the protein to DNA ratio of the rudiments increases instead to 50 (Table I).

Published November 1, 1977

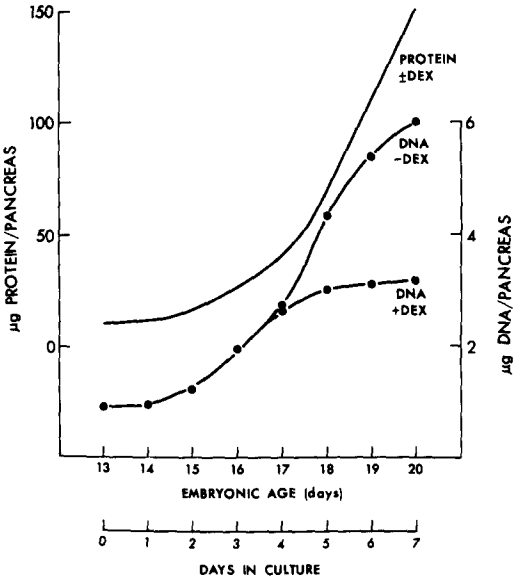


FIGURE 1 Effect of dexamethasone on the accumulation of DNA and protein in the developing rat pancreas in vitro. Day-13 pancreatic rudiments, including most of the associated mesenchyme, were excised and cultured in the presence and absence of 10^{-7} M dexamethasone. The samples were harvested at the indicated intervals and assayed for protein and DNA content. The control values represent an average of from three independent determinations early in development to ten later in development. They vary by no more than 10%. One steroid series was assayed except the 20-day results which are an average of five different experiments. In total, glucocorticoids inhibit one round of DNA replication and the effect is first observed after 4 days in culture. At the same time, protein accumulation continues unabated.

Glucocorticoids Affect the Cellular Levels of Several Exocrine Enzymes

The exocrine enzymes of the pancreas accumulate to high levels in vitro as in vivo (19, 43). Their specific activities based on total protein increase by three orders of magnitude between days 13 and 20 and, with the exception of procarboxypeptidase B, the differentiated levels are simlar whether expressed in 20-day rudiments or in 13-day pancreases after 7 days in culture (Table I). However, as shown in Table I, the total protein content per differentiated cell in vitro is only one-third of that in vivo. Therefore, with the exception of procarboxypeptidase B, the in vitro specific activities of the cell-specific proteins expressed on a DNA basis are about one-third of the in vivo values.

When dexamethasone (10^{-7} M) is included in the medium, the final cellular levels of amylase and procarboxypeptidase B are stimulated about 10-fold. Those of chymotrypsinogen and procarboxypeptidase A are increased twofold while lipase A remains unchanged (Table I). The substantial increase in the cellular levels of amylase and chymotrypsinogen, the two major proteins synthesized by the pancreas, accounts for the observed shift in the total protein to DNA ratio. Thus, by difference it can be calculated that amylase and chymotrypsinogen contribute an additional 19 and 6 μg of protein, respectively, to account for the overall 25 μg of protein per μg of DNA increase elicited by dexamethasone. Other pancreatic-specific products represent a small proportion of the total protein content of the pancreas and therefore, in the presence or absence of dexamethasone, the cellular content of a variety of other cell proteins (called in this publication nonspecific proteins) is quantitatively similar.

These results are found at concentrations of dexamethasone that are effective in other systems. Fig. 2 shows that a significant stimulation of amylase content is observed at 10^{-9} M dexamethasone; the half maximal effect occurs at 10^{-8} M and the maximal effect at 10^{-7} M. Hydrocortisone gives a similar response, but at 50-fold higher concentrations. In all subsequent experiments, dexamethasone, when added, was present at 10^{-7} M.

Glucocorticoids Do Not Precociously Induce Cell-Specific Protein Accumulation

Dexamethasone, while dramatically stimulating amylase levels during development, does not precociously induce the appearance of amylase. In the developing rat pancreas, amylase, like the other exocrine enzymes and insulin, accumulates in a biphasic pattern (30, 31, 34, 40). The secondary increase (days 13–18) is exponential, changing by an order of magnitude every 2 days in vitro. As shown in Fig. 3, dexamethasone stimulates this rate by about 30%, which results in the fourfold increase in the final amylase concentration (based on total pancreatic protein). However, both accumulation profiles extrapolate to the same point and therefore, there is no significant change in the time of the initial onset of amylase accumulation.

Published November 1, 1977

TABLE I

Glucocorticoids Modulate the In Vitro Development of the Embryonic Rat Pancreas

Macromolecule	In vivo	In vitro	
		−Dex	+Dex
μg Protein/pancreas	3,300	150	150
μg DNA/pancreas	49	6	3
Protein/DNA ratio	67	25	50
Amylase	8.7 (13)	2.8 (11)	21.5 (43)
Procarboxypeptidase B	0.56 (0.83)	0.025 (0.10)	0.35 (0.70)
Chymotrypsinogen	20 (30)	6.5 (26)	13 (26)
Procarboxypeptidase A	2.0 (3.0)	0.58 (2.3)	1.2 (2.4)
Lipase	0.07 (0.10)	0.03 (0.12)	0.03 (0.06)
Insulin	0.04 (0.06)	0.07 (0.28)	0.03 (0.06)
Cell-specific protein/DNA	31	10	36
Nonspecific protein/DNA	36	15	14

Day-13 pancreatic rudiments were grown for a period of 7 days in the presence of 10^{-7} M dexamethasone. Total protein, enzyme, and hormone levels are expressed as μg/μg DNA . Units of enzyme activity have been converted to absolute levels, knowing the turnover numbers of the various enzymes (see Materials and Methods). Numbers in parentheses are the same data expressed on a protein basis and multiplied by 100, that is, percent of total pancreatic protein. The higher insulin specific concentration in pancreases grown in vitro as opposed to in vivo is due to a different effect of nutritional conditions on exocrine and endocrine development. However, the effect of dexamethasone is independent of these nutritional conditions. The analysis of these observations will be the subject of another report. Protein, DNA, amylase, and insulin values represent an average of 10 (in vitro minus dexamethasone) and 5 (in vitro plus dexamethasone and in vivo) independent determinations. Other enzyme levels are the average of two experiments and in all cases, the data vary by no more than 10%.

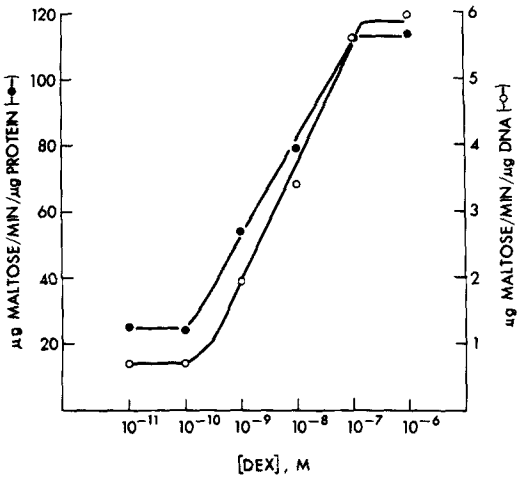


FIGURE 2 Effect of increasing concentrations of dexamethasone on the specific activity of amylase in the late embryonic pancreas in vitro. Day-13 pancreases were cultured for 7 days and then the amylase, protein, and DNA content were measured. The results are expressed as micrograms of maltose released per minute per microgram of protein or DNA. In both cases half-maximal effects are achieved between 0.5 and 1.0 × 10^{-8} M dexamethasone.

Glucocorticoids Elicit Ultrastructural Changes Reflecting an Increased Capacity for the Synthesis and Packaging of Acinar Proteins

The normal morphological development of the exocrine pancreas involves a rapid proliferation of the rough endoplasmic reticulum and the subsequent appearance of the first zymogen granules between days 16 and 17 in vivo or in pancreatic rudiments explanted on day 13, after 3–4 days in culture (31). With the exception of a lower cytoplasmic-nuclear ratio, there are few ultrastructural differences between the cells in vitro and in vivo. However, if dexamethasone is included in the culture medium, there is an obvious acceleration of cytodifferentiation in acinar cells. Thus, typically, by day 17 in vivo many of the acinar cells contain at least a few zymogen granules which are concentrated in the apical region of the cell. The rough endoplasmic reticulum is oriented parallel to the circumference of the nucleus, but is not yet extensive or laminated. In contrast, at the same stage of development in vitro in the presence of dexamethasone, the cytoplasmic distribution and content of

Published November 1, 1977

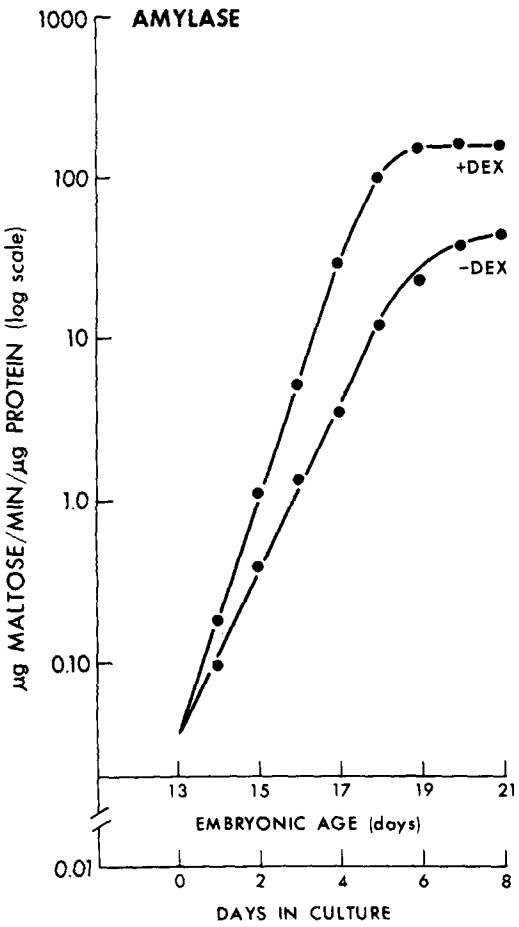


FIGURE 3 Effect of dexamethasone on the accumulation of amylase during pancreatic development in vitro. Day-13 pancreatic rudiments were excised and cultured in the presence and absence of 10^{-7} M dexamethasone. On a daily basis, rudiments were harvested and then assayed for amylase and protein content. The results are expressed as micrograms of maltose released per minute per microgram of protein. The conditions and the number of samples as well as the variation in the data are as in the legend to Fig. 1. To insure uniform and extensive growth, the early pancreas is cultured in association with large amounts of mesenchymal tissue. Early in the culture period, then, the specific activity of amylase, an epithelial product, is artificially lowered. Therefore, to more accurately determine its activity in these samples (day 13 plus 1 day in culture only), the mesenchyme was mostly excluded.

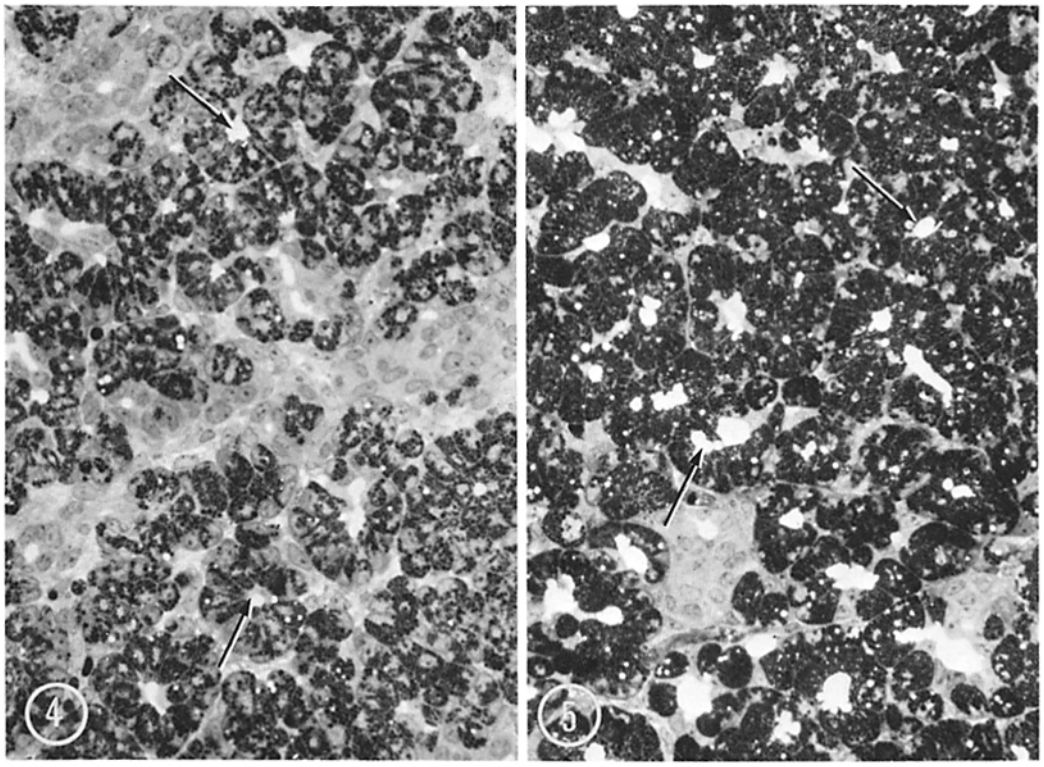
rough endoplasmic reticulum is more characteristic of acinar cells one day later in development. By day 19 or 20, when cytodifferentiation is complete, a light microscope comparison of control (Fig. 4) and dexamethasone-treated rudiments (Fig. 5) shows an enhanced number of zymogen granules. Electron microscopically, this is correlated with an increase in the amount of rough endoplasmic reticulum and in the size of the nucleoli and, in agreement with these observations, the final cellular content of RNA (mostly ribosomal) is stimulated approx. twofold (3.8 vs. 2.0 μg RNA/μg DNA).

An unexpected and striking feature of dexamethasone-treated tissues is shown in Figs. 6–9. Many regions of the rough endoplasmic reticulum in the acinar cells are dilated and contain partially condensed material. The cytoplasm of many of these cells contains linear arrays of a material with an electron density similar to that found in zymogen granules (Fig. 6). The number and size of these inclusions vary greatly and they are often distributed in parallel stacks (Fig. 7). These inclusions are located in the cisternae of the rough endoplasmic reticulum (Fig. 8) including the perinuclear cisternae (Fig. 9) and sometimes appear crystalline (Fig. 9). These structures are found only in pancreases cultured in the presence of dexamethasone; we have never seen them in control pancreases in vitro or in vivo.

Glucocorticoids Inhibit Cell Division and Decrease Disproportionately the Gland Content of Insulin

At the same levels at which it potentiates exocrine cell development, dexamethasone inhibits cell division. As shown in Fig. 1, between days 13 and 20 in control cultures, there is a six- to sevenfold increase in the amount of DNA per rudiment. If dexamethasone is included in the culture medium, the DNA content of the pancreas increases only by about 3.5-fold. This inhibition of DNA accumulation is first observed after 4 days in culture when an average of 1.5 rounds of DNA replication has occurred. Apparently, then, the last cell divisions of the culture period are blocked by the action of dexamethasone. In contrast, over the same time period, protein accumulation continues unabated.

If the relative number of insulin-producing cells in cultured rudiments is not altered by the presence of dexamethasone, then one might expect to find a twofold lower gland content of hormone in proportion to the smaller total cell number. In fact, as calculated from the data presented in Table 1, there is a disproportionate fivefold lower insulin content per differentiated pancreas. These



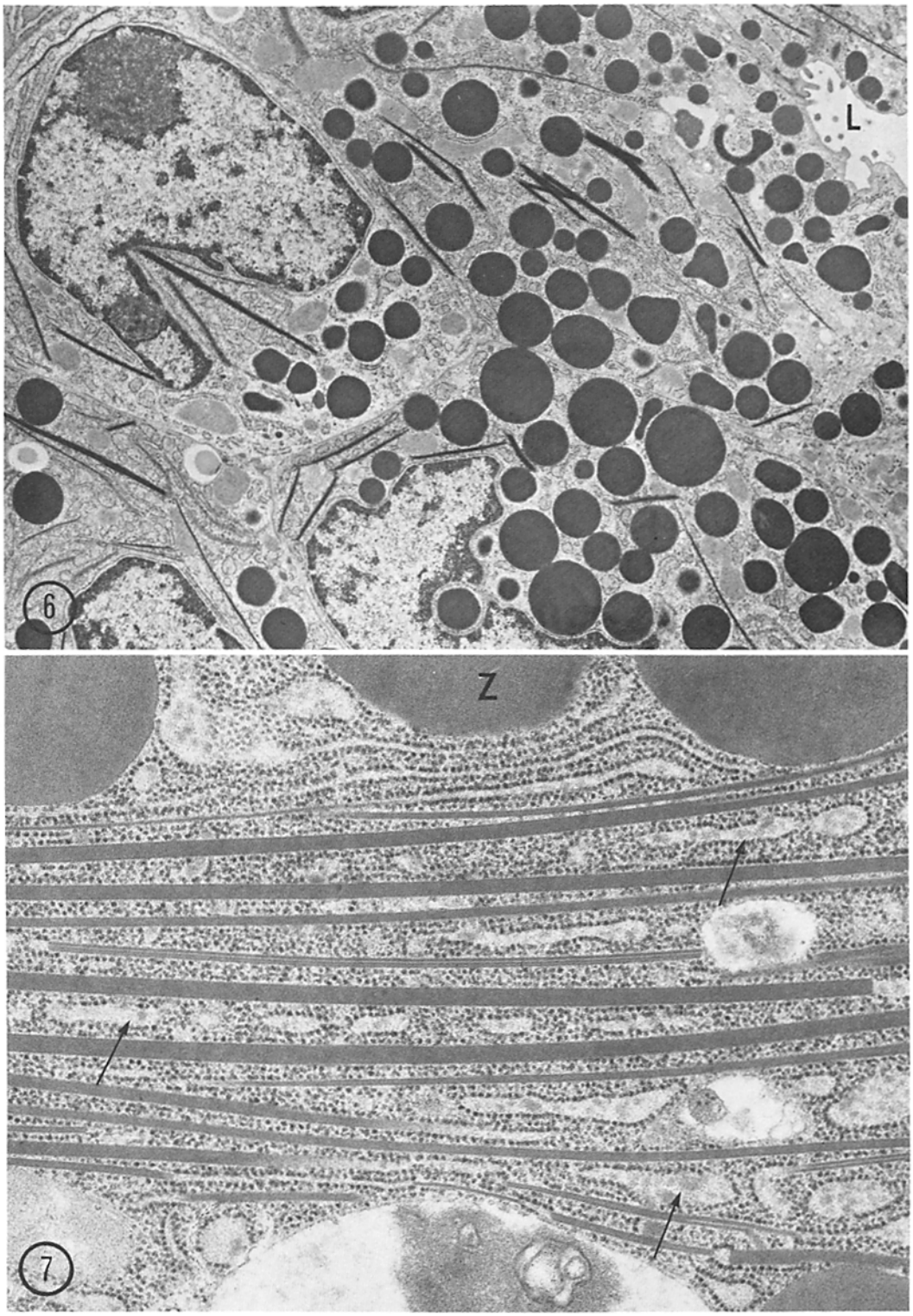
FIGURES 4 and 5 Acinar cells of dexamethasone-treated pancreatic rudiments accumulate more zymogen granules. Embryonic pancreases were explanted on day 13 of gestation and cultured in the absence (Fig. 4) and presence (Fig. 5) of 10^{-7} M dexamethasone. In both cases, morphogenetic development is normal and acini (arrows) are present. In the steroid-treated tissue, however, acinar cell cytoplasm is darker due to the higher content of zymogens. Both × 325.

changes might be brought about by a reduction in the insulin content per cell or in the number of B cells present in the tissue. Although quantitative morphometry has not been carried out, dexamethasone must at least decrease insulin synthesis within individual cells. First, other experiments (M. de Gasparo and R. L. Pictet, unpublished observations) have shown no effect of glucocorticoids on insulin secretion in our in vitro system. Second, the addition of glucocorticoids to the culture medium later in development when B cell precursors are no longer proliferating reduces the hormone content per rudiment by a factor of 2 (33).

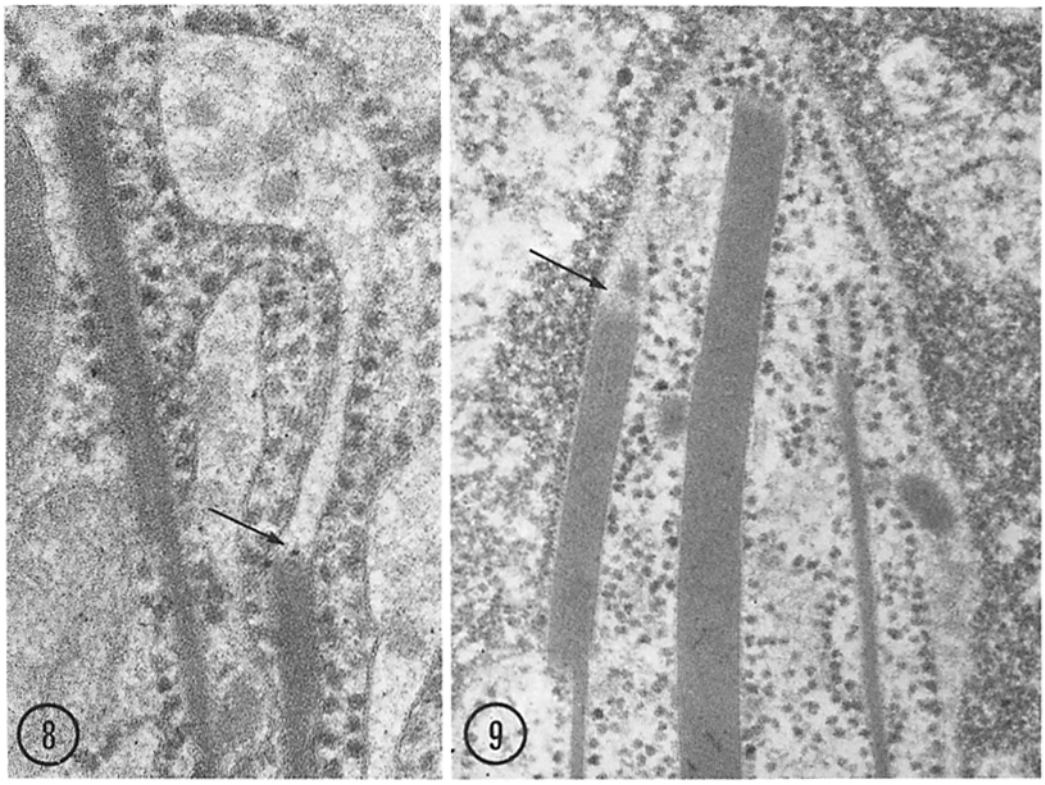
Glucocorticoids Are Not Obligatory for Pancreatic Cytodifferentiation

Although dexamethasone exerts specific developmental effects on the rat pancreas, it is apparently not obligatorily required for the process of cytodifferentiation or the accumulation of the cell-specific proteins. It is of course difficult to assess the contribution of glucocorticoids derived from the maternal circulation. However, the rat pancreas forms midway through gestation (day 11), 2 days before the appearance of the adrenal cortex (38) and, therefore, at a time when no embryonically synthesized adrenal cortical hormones can be present. Rudiments excised at this early stage accumulate amylase and insulin to high levels (Table II and reference 45) during cultivation in a defined medium devoid of any steroids. This was accomplished by eliminating embryo extract or bovine serum albumin which contain low amounts of glucocorticoids (2×10^{-9} M and 4×10^{-11} M in 10% embryo extract and 0.1% bovine serum albumin solutions, respectively). In addition, differentiated (granule-containing) acinar and endocrine B cells are readily identifiable in electron micrographs.

Published November 1, 1977



FIGURES 6 and 7 The cytoplasm of glucocorticoid-stimulated acinar cells contains crystalline-shaped, electron-dense material. Day-13 pancreases were cultured for 7 days in the presence of dexamethasone (10^{-7} M).The differentiated level of several exocrine enzymes is increased and at the same time a variable number of cells contains, in addition to the zymogen granules, an electron-dense material (Fig. 6) which often is distributed in long, linear, parallel stacks (Fig. 7). This is a striking unusual feature of glucocorticoid-treated samples; it is never observed in vivo or in vitro in the absence of dexamethasone. Fig. 6, × 7,500; Fig. 7, × 35,000.



FIGURES 8 and 9 Details of dexamethasone-induced, crystalline material. Day-13 pancreases were cultured for 7 days in the presence of dexamethasone (10^{-7} M). In the presence of glucocorticoids, regions of the acinar cell rough endoplasmic reticulum are dilated and contain partially condensed material which is in direct continuation with the fully condensed material (Fig. 8).This material sometimes shows a clear crystalline pattern (Fig. 9). Fig. 8, × 123,500; Fig. 9, × 49,500.

TABLE II

Development of the Exocrine and Endocrine Rat Pancreas in a Completely Defined Medium

	Amylase		Insulin	
Embryonic age	sp act	Increase over day-13 level in vivo	sp act	Increase over day-13 level in vivo
Day 11 + 9 days in culture	5.4 μg maltose equivalent/μg protein	1,000-fold	2.92 ng/μg protein	1,500-fold

Day-11 rudiments at the onset of pancreatic organogenesis were excised and cultured under standard conditions. The medium was not supplemented with a protein source. After 9 days, amylase and insulin levels were measured. These values are minimal since nonpancreatic tissues are included in the sample. They are compared with the low levels found in the early 13-day pancreas: amylase, 0.005 μg maltose equivalent per min per μg protein, and insulin, 0.002 ng per μg protein. Day-11 activities have not been similarly determined.

DISCUSSION

The exocrine enzymes of the pancreas and insulin accumulate to high levels in vivo and similarly in vitro (19, 33, 43). However, even under enriched culture conditions, the cellular content of the exocrine enzymes does not reach in vivo values. Nevertheless, the proportion of cell-specific proteins relative to total protein is similar in the two circumstances (~50%) (see Table I). Also, the

relative proportions of the two major pancreatic proteins, amylase and chymotrypsinogen, and of two minor proteins, procarboxypeptidase A and lipase A, are maintained. The differentiated level of procarboxypeptidase B is an exception; expressed on the basis of total protein, it is lower in vitro by an order of magnitude.

Differentiated exocrine cells in vitro contain about a third of the total protein of exocrine cells in vivo. This indicates that the exocrine cells of the pancreas developing in vitro are smaller than those in vivo. Morphological observations, although not quantitative, are consistent with this view; that is, granule-containing exocrine cells are larger in size than their precursor cells, and the increased size is not so obvious in pancreases that develop in vitro.

In the presence of low levels of dexamethasone, neither the final cell size as seen by microscopy nor the cellular levels of nonspecific proteins are appreciably altered, but there is a significant increase in several exocrine enzymes. The total zymogen content per cell late in development in vitro is stimulated three- to fourfold and approaches in vivo values. This effect is largely due to a 10-fold increase in amylase content and a twofold increase in chymotrypsinogen content. Other cell-specific proteins that are present at much lower levels are variously affected. Procarboxypeptidase B levels are increased 10-fold, procarboxypeptidase A levels, twofold, and lipase A is unaffected. Of the five enzymes measured, three of them (procarboxypeptidases A and B and chymotrypsinogen) reach levels more closely approaching those found in vivo. However, amylase levels exceed in vivo values by more than twofold. In essence, the presence of glucocorticoids alters the intrinsic ratio of one enzyme to another and increases the proportion of pancreatic-specific proteins from 50% to 70% of the total.

This selective stimulation of cell-specific proteins, especially amylase, may be the cause of the formation of crystals within the rough endoplasmic reticulum. Similar crystalline arrays have been observed after the injection of *p*-chlorophenylalanine (100 mg/kg). However, in this case crystals form within minutes and are also present in the duct lumen (11, 12). The mechanism of this intriguing effect of *p*-chlorophenylalanine is unknown, but is likely to be different from that documented here.

Glucocorticoid action is not restricted to mammalian pancreatic development. Cohen et al. (8) have shown that hydrocortisone elicits increases in the levels of certain zymogens, especially chymotrypsinogen, as well as marked cytological changes in the developing chick pancreas. The cytological effects of glucocorticoids involve the enhanced production of cellular organelles required for the synthesis of large quantities of exportable proteins. They have also been observed in the response of the developing mammary gland to hydrocortisone (24) and in our cultured rat pancreases. That is, in the presence of dexamethasone, the elaboration of the rough endoplasmic reticulum is more extensive and occurs earlier in development. The nucleolus is enlarged and after 7 days in culture there is twice as much total RNA per cell. These changes are certainly not, however, the sole basis for the glucocorticoid response since they would be expected to uniformly enhance the synthesis of the exocrine enzymes (29). It has been suggested that glucocorticoids may alter enzyme levels by in part reducing the rate of protein (35) or mRNA turnover (46). We have previously shown that the secretory products of the developing rat pancreas turn over slowly, if at all (19, 33), and their mRNA's, as in other developing systems (4, 29), appear to be relatively stable. Therefore, in this system glucocorticoids may selectively increase the number of certain mRNA molecules. Preliminary evidence from our laboratory supports this contention (J. D. Harding, R. J. MacDonald, A. E. Przybyla, J. M. Chirgwin, R. Pictet, and W. J. Rutter, unpublished observations).

In addition to their well-studied induction of tyrosine aminotransferase message (46), glucocorticoids have been shown to suppress DNA synthesis in cell lines of liver origin (22). We also observe such an inhibition. In the normal development of the pancreas in vivo, there is about a 50-fold increase in the total cell number between days 13 and 20 (33). During the same period in vitro there is only a six- to sevenfold change. In the presence of dexamethasone, there is an even lower three- to fourfold increase in cell number. The DNA accumulation profiles show that this effect is not immediate and may reflect a different mechanism of control of cell proliferation early and late in embryonic development.

Several studies on adult and neonatal rats suggest that glucocorticoids also produce effects on the exocrine pancreas similar to those described here. After cortisone administration to neonatal rats, the total pancreas RNA content, the size of

the nucleolus, and the number of zymogen granules per cell are increased. In addition, elevated levels of pancreatic amylase and trypsin-like activity are detected (44). The timing of the formation of the adrenal cortex is compatible with a role of glucocorticoids in certain stages of pancreatic development. In the rat, the organization of the adrenal cortex begins on day 13 (38), 2 days after the initial formation of the pancreatic diverticulum. The adrenal gland content (18) and the circulating levels (7) of corticosterone, the principal glucocorticoid of the rat, reach adult levels by day 17. Thus, corticosterone of embryonic origin is present at high levels at the time of the dramatic accumulation of the exocrine enzymes which occurs between days 15 and 20. In spite of this, the relative proportion of the various zymogens in the in vivo embryonic pancreas is different from the proportion found after cultivation with dexamethasone. Instead, in fact, they are similar when the pancreas is grown in the absence of glucocorticoids. In addition, the morphological changes produced by glucocorticoids are never observed in vivo.

The difference in the proportion of zymogens in vivo and in vitro in the presence of glucocorticoids may be due to (*a*) the absence of glucocorticoid receptors in the pancreas in vivo. If this were the case, our results would require the rapid induction of these receptors under our in vitro conditions. (*b*) Other hormones might act in concert with glucocorticoids to balance the synthesis of the cell-specific products or (*c*) glucocorticoid action may be antagonized in the fetus. The latter explanation is favored since it is known that enzymes induced by glucocorticoids in the adult or neonatal rat are relatively insensitive to glucocorticoids administered to the fetus (14). On the other hand, the fetal tissues extirpated and cultivated in vitro respond to the hormone (23).

The relatively late appearance of glucocorticoids plus the results of our in vitro experiments indicating that these steroids are not obligatorily required for cytodifferentiation and the accumulation of high levels of the cell-specific products support the contention that glucocorticoids do not play an instructive role, but rather may play a modulatory role in the development of the pancreas.

We would like to thank Ms. J. Carter and Dr. P. L. Ballard for measuring glucocorticoid levels in embryo extract and in albumin solutions; and Ms. Jennifer Meek for technical assistance.

This research was supported by grants from the National Foundation–March of Dimes and the National Science Foundation (BMS72-02222). R. Pictet is a recipient of a National Institutes of Health Career Development Award.

Received for publication 28 February 1977, and in revised form 11 July 1977.

REFERENCES

1. Ballard, P. L., and R. A. Ballard. 1972. Glucocorticoid receptors and the role of glucocorticoids in fetal lung development. *Proc. Natl. Acad. Sci. U. S. A.* **69:**2668–2672.
2. Bernfeld, P. 1955. Enzymes of carbohydrate metabolism: amylases, α and β. *Methods Enzymol.* **1:**149–150.
3. Bradshaw, W. S., and W. J. Rutter. 1972. Multiple pancreatic lipases. Tissue distribution and pattern of accumulation during embryological development. *Biochemistry.* **11:**1517–1527.
4. Buckingham, M. E., D. Caput, A. Cohen, R. G. Whalen, and F. Gros. 1974. The synthesis and stability of cytoplasmic messenger RNA during myoblast differentiation in culture. *Proc. Natl. Acad. Sci. U. S. A.* **71:**1466–1470.
5. Burton, K. 1968. Determination of DNA concentration with diphenylamine. *Methods Enzymol.* **12:**163–166.
6. Clark, W. R., and W. J. Rutter. 1972. Synthesis and accumulation of insulin in the fetal rat pancreas. *Dev. Biol.* **29:**468–481.
7. Cohen, A. 1973. Plasmacorticosterone concentration in the foetal rat. *Horm. Metab. Res.* **5:**66.
8. Cohen, A., H. Heller, and R. G. Kulka. 1972. The effect of hydrocortisone on exportable enzyme accumulation in the differentiating chick pancreas. *Dev. Biol.* **29:**293–306.
9. Dische, Z. 1955. Color reactions of nucleic acid components. *In* The Nucleic Acids. Vol. I. E. Chargaff and J. N. Davidson, editors. Academic Press, Inc., New York. 300–302.
10. Eisen, H. J., I. D. Goldfine, and W. H. Glinsman. 1973. Regulation of hepatic glycogen synthesis during fetal development: Roles of hydrocortisone, insulin and insulin receptors. *Proc. Natl. Acad. Sci. U. S. A.* **70:**3454–3457.
11. Forssmann, W. G., and W. Bieger. 1973. Biosynthese falscher proteine: Einbau von *p*-chlorophenylalanin in enzyme des exokrinen pankreas. *Res. Exp. Med.* **160:**1–20.
12. Forssmann, W. G., W. Bieger, and H. Roth. 1973. Untersuchungen der falschen proteinsynthese im exokrinen pankreas. *Verh. Anat. Ges.* **67:**507–515.
13. Giannopoulos, G., Z. Hassan, and S. Solomon. 1974. Glucocorticoid receptors in fetal and adult rabbit tissues. *J. Biol. Chem.* **249:**2424–2427.

14. GREENGARD, O., and H. K. DEWEY. 1970. The premature deposition or lysis of glycogen in livers of fetal rats injected with hydrocortisone or glucagon. *Dev. Biol.* **21:**452-461.
15. HENNING, S. J., P. L. BALLARD, and N. KRETCHMER. 1975. A study of the cytoplasmic receptors for glucocorticoids in intestine of pre- and postweanling rats. *J. Biol. Chem.* **250:**2073-2079.
16. JONES, R. E., and A. A. MOSCONA. 1974. Effects of cytosine arabinoside on differential gene expression in embryonic neural retina. I. Accumulation of glutamine synthetase with suppression of macromolecular synthesis. *J. Cell Biol.* **61:**688-700.
17. JOST, A. 1971. Hormones in development: Past and prospects. *In* Hormones in Development. M. Hamburgh and E. J. W. Barrington, editors. Appleton-Century-Crofts, New York. 1-18.
18. KAMOUN, A., C. Mailhe-Voloss, and F. STUTINSKY. 1964. Evolution de la tenure en corticosterone de la surrenale foetale du rat. *C. R. Soc. Biol.* **158:**828-832.
19. KEMP, J. D., B. T. WALTHER, and W. J. RUTTER. 1972. Protein synthesis during the secondary developmental transition of the embryonic rat pancreas. *J. Biol. Chem.* **247:**3941-3952.
20. LIN, R. I., and O. A. SCHJEIDE. 1969. Micro estimation of RNA by cupric ion catalyzed orcinol reaction. *Anal. Biochem.* **27:**473-483.
21. LIPPMAN, M. E., B. O. WIGGERT, G. J. CHADER, and E. B. THOMPSON. 1974. Glucocorticoid receptors. Characteristics, specificity and ontogenesis in the embryonic chick neural retina. *J. Biol. Chem.* **249:**5916-5917.
22. LOEB, J. N., C. BOREK, and L. L. YEUNG. 1973. Suppression of DNA synthesis in hepatoma cells exposed to glucocorticoid hormone *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **70:**3852-3856.
23. LUPPIS, B., and W. J. RUTTER. 1973. Induction of tyrosine aminotransferase by triamcinolone during rat liver development *in vitro*. *Dev. Biol.* **33:**482-486.
24. MILLS, E. S., and Y. J. TOPPER. 1970. Some ultrastructural effects of insulin, hydrocortisone and prolactin on mammary gland explants. *J. Cell Biol.* **44:**310-328.
25. MOOG, F. 1971. Corticoids and the enzymic maturation of the intestinal surface: Alkaline phosphatase, leucyl naphthylamidase, and sucrase. *In* Hormones in Development. M. Hamburgh and E. J. W. Barrington, editors. Appleton-Century-Crofts, New York. 143-160.
26. MOOG, F., A. E. DENES, and P. M. POWELL. 1973. Disaccharidases in the small intestine of the mouse: Normal development and influence of cortisone, actinomycin D and cycloheximide. *Dev. Biol.* **35:**143-159.
27. MORGAN, C. R., and A. LAZAROW. 1963. Immunoassay of insulin: two antibody systems. Plasma insulin levels of normal, subdiabetic and diabetic rats. *Diabetes.* **12:**115-126.
28. MOSCONA, A. A. 1971. Control mechanisms in hormonal induction of glutamine synthetase in the embryonic neural retina. *In* Hormones in Development. M. Hamburgh and E. J. W. Barrington, editors. Appleton-Century-Crofts, New York. 169-189.
29. PALMITER, R. D. 1975. Quantitation of parameters that determine the rate of ovalbumin synthesis. *Cell.* **4:**189-197.
30. PICTET, R., and W. J. RUTTER. 1972. Development of the embryonic endocrine pancreas. *In* Handbook of Physiology. Section 7: Endocrinology. Vol. I. Endocrine Pancreas. N. Freinkel and D. F. Steiner, editors. Williams and Wilkins, Baltimore. 25-66.
31. PICTET, R., W. R. CLARK, R. H. WILLIAMS, and W. J. RUTTER. 1972. An ultrastructural analysis of the developing embryonic pancreas. *Dev. Biol.* **29:**436-467.
32. PLAS, C., F. CHAPEVILLE, and R. JACQUOT. 1973. Development of glycogen storage ability under cortisol control in primary cultures of rat fetal hepatocytes. *Dev. Biol.* **32:**82-91.
33. RALL, L. B. 1975. On the development of the embryonic rat pancreas. Doctoral thesis. University of Washington, Seattle.
34. RALL, L. B., R. L. PICTET, R. H. WILLIAMS, and W. J. RUTTER. 1973. Early differentiation of glucagon-producing cells in embryonic pancreas: a possible developmental role for glucagon. *Proc. Natl. Acad. Sci. U. S. A.* **70:**3478-3482.
35. REEL, J. R., K.-L. LEE, and F. T. KENNEY. 1970. Regulation of tyrosine α-ketoglutarate transaminase in rat liver. VIII. Inductions by hydrocortisone and insulin in cultured hepatoma cells. *J. Biol. Chem.* **245:**5800-5805.
36. REIF-LEHRER, L. 1968. Development of glutamine synthetase activity in chick embryo retina cultures in the absence of added hydrocortisone. *Biochem. Biophys. Res. Commun.* **33:**984-989.
37. RONZIO, R. A., and W. J. RUTTER. 1973. Effects of a partially-purified factor from chick embryos on macromolecular synthesis of embryonic pancreatic epithelia. *Dev. Biol.* **30:**307-320.
38. RUGH, R. 1968. The Mouse, Its Reproduction and Development. Burgess Publishing Co., Minneapolis.
39. RUTTER, W. J. 1967. Protein determinations in embryos. *In* Methods in Developmental Biology. N. K. Wessells and F. A. Wilt, editors. Thomas Y. Crowell Co., New York. 671-683.
40. RUTTER, W. J., J. D. KEMP, W. S. BRADSHAW, W. R. CLARK, R. A. RONZIO, and T. G. SANDERS. 1968. Regulation of specific protein synthesis in cytodifferentiation. *J. Cell. Physiol.* **72**(Suppl. 1):1-18.
41. SANDERS, T. G. 1970. Purification, properties and

embryonic regulation of pancreatic amylolytic and proteolytic enzymes of the rat. Doctoral Thesis. University of Illinois, Urbana, Ill.

42. SANDERS, T. G., and W. J. RUTTER. 1972. Molecular properties of rat pancreatic and parotid α-amylase. *Biochemistry*. **11:**130–136.
43. SANDERS, T. G., and W. J. RUTTER. 1974. The developmental regulation of amylolytic and proteolytic enzymes in the embryonic rat pancreas. *J. Biol. Chem.* **249:**3500–3509.
44. SESSO, A. 1962. Effect de la thyroxine et de la cortisone sur les grains de secretion, les acides nucleiques et les activites amylolytiques et proteolitiques du pancreas chez le jeune rat. *C. R. Acad. Sci. (Paris)*. **254:**269–570.
45. SPOONER, B. S., B. T. WALTHER, and W. J. RUTTER. 1970. The development of the dorsal and ventral mammalian pancreas *in vivo* and *in vitro*. *J. Cell Biol.* **47:**235–246.
46. STEINBERG, R. A., B. B. LEVINSON, and G. M. TOMKINS. 1975. Kinetics of steroid induction and deinduction of tyrosine aminotransferase synthesis in cultured hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **72:**2007–2011.
47. SUGIMOTO, M., K. TAJIMA, A. KOJIMA, and H. ENDO. 1974. Differential acceleration by hydrocortisone of the accumulation of epidermal structural proteins in the chick embryonic skin growing in a chemically defined medium. *Dev. Biol.* **39:**295–307.
48. TOPPER, Y. J., and B. K. VONDERHAAR. 1974. The role of critical cell proliferation of mammary epithelial cells. *In* Control of Proliferation in Animal Cells. Cold Spring Harbor Conferences on Cell Proliferation. Vol. I. B. Clarkson and R. Baserga, editors. Cold Spring Harbor Press, Cold Spring Harbor, N. Y. 843–852.