5-BROMODEOXYURIDINE MAY ALTER THE DIFFERENTIATIVE PROGRAM OF THE EMBRYONIC PANCREAS

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ABSTRACT

The thymidine analog, 5-bromodeoxyuridine (BrdU), inhibits the differentiation of the acinar cells of the embryonic rat pancreas, while having little effect on the growth of the tissue. The BrdU-treated pancreas contains elevated alkaline phosphatase and carbonic anhydrase activities, and, unlike the normal pancreas, contains numerous extracellular fluid-filled vacuoles, surrounded by ductlike cells. Both alkaline phosphatase and carbonic anhydrase activities are located preferentially in the ductlike cells lining the vacuoles. The biochemical, morphological, and functional features of these epithelial cells are therefore characteristic of the normal pancreatic duct cell. Thus, in the exocrine pancreas, BrdU seems to alter the normal program of differentiation by favoring the formation of functional duct cells while inhibiting the differentiation of acinar cells.

The thymidine analog, 5-bromodeoxyuridine (BrdU), inhibits differentiation in a number of systems. This agent blocks the synthesis of cell specific products without significantly affecting growth (1, 5, 11, 31, 51, 54, 55, 58, 59, 60, 62).

In our previous studies on the development of the embryonic pancreas in vitro, we have shown that BrdU selectively inhibits the synthesis of the exocrine proteins at concentrations that have little if any effect on general protein, RNA or DNA synthesis or on the principal metabolic pathways (55). We also observed that these BrdU-treated pancreases develop large fluid-filled vacuoles linked by cells which appear similar to duct cells (55). We have further tested the possibility that these cells are in fact duct cells, by measuring the levels of alkaline phosphatase and carbonic anhydrase, enzymes which are characteristic for these cells: both of these enzymes are associated with tissues active in fluid transport (23, 35); and in the pancreas they are localized primarily in the duct cells (10, 21, 36, 56). The activities of these enzymes have been determined by conventional enzymatic methods, and their cellular location has been visualized by specific histochemical procedures. We have found that BrdU induces both alkaline phosphatase and carbonic anhydrase coincident with the formation of vacuoles. Both of the induced enzymes are localized preferentially in the epithelial cells lining the vacuoles. Thus in the presence of BrdU, cultured embryonic pancreases contain many cells that by ultrastructural, biochemical, and functional criteria are identical to normal duct cells. BrdU therefore appears to enhance dramatically the proportion of duct cells in the rudiment. This result is consistent with the existence of a pluripotent cell that is a precursor of the acinar cells, and may be equivalent to, or

converted to, the duct cell. Thus BrdU acts to prohibit the differentiation of acinar cells, and favor the formation of duct cells.

MATERIALS AND METHODS

Organ Culture

Embryonic rat pancreases were removed on the beginning of day 14 of gestation and were maintained in organ culture as described previously (48). The culture medium contained 90% Eagle's basal medium supplemented with seven times the concentration of essential amino acids, 10% chick embryo extract (12), 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml of Fungizone (E. R. Squibb & Sons, New York, N. Y.). In some experiments, pancreatic rudiments were isolated from 12-day embryos and were deprived of their mesenchyme by trypsin treatment (48). The resulting pancreatic buds were cultured in the above medium supplemented with 10% mesenchymal factor (30) in place of embryo extract. In other experiments, sections (about 1.5 mm) of the 14-day duodenum (dissected on the point of entry of the pancreatic duct) were cultured in parallel with 14-day pancreatic rudiments as described above.

Preparation of Tissues for Assays

Adult pancreatic islets were isolated by collagenase digestion (28). A preparation enriched in adult ducts was obtained by dissection after visualization of the major ducts by the retrograde injection of Trypan blue after cannulation of the biliary duct. The embryonic pancreases and duodenum sections, and the pancreatic islets and ducts were homogenized by sonication (55). There is no loss of activity by sonication as compared to homogenization of the tissue by a Potter-Elvehjem homogenizer.

Adult tissues (pancreas and duodenum) were obtained from freshly sacrificed animals, minced in water at 0°C, homogenized in a motor-driven Teflon glass homogenizer and sonicated for 15 s to provide a thoroughly disrupted tissue preparation. Chemical and enzymatic analyses were performed on the entire sonicate in all cases, rather than on a supernatant fraction, since alkaline phosphatase is associated, at least in part, with rapidly sedimenting material.

Enzyme and Chemical Assays

Alkaline (pH 10), neutral (pH 7), and acid (pH 5) phosphatase activities were measured in the presence of 10 mM *p*-nitrophenylphosphate, 10 mM $MgCl_2$, 0.1% Triton X-100, 0.1% bovine serum albumin (33), and 50 mM piperazine + 50 mM glycylglycine at the appropriate pH, in an assay volume of 0.1 ml at 37°C. The reactions were stopped by the addition of 0.2 ml of 0.1 N NaOH, and the tubes were chilled and centrifuged to remove the slight turbidity which was frequently present. The optical density was then measured at 400 nm. The molar extinction coefficient of the product, *p*-nitrophenol, was taken to be 17×10^3 at 400 nm. In some experiments, β-glycerophosphate was used as a substrate for the phosphatase activity and the released P_i was assayed (9). (Na + K)-ATPase (44), Ca-ATPase (47), and HCO_3-ATPase (52) (all of which are neutral pH ATPase activities stimulated by the indicated cations or anions) were also measured. 1 U of phosphatase activity was defined as that amount of enzyme producing 1 μmol of product per minute. Catalase (2), amylase (3), DNA (6), and protein (49) were assayed by the indicated methods. 1 U of amylase was defined as releasing 1 mg of maltose per minute.

Polyacrylamide Gel Electrophoresis of Alkaline Phosphatase

Alkaline phosphatase was solubilized according to the procedure of Morton (39), and 50–75% of the activity of the original sonicate was recovered in the aqueous extract. The extracts were desalted by passage through a short column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in water at room temperature. Fractions containing alkaline phosphatase activity were concentrated by freeze-drying and resuspended in a small vol of 1% Triton X-100 + 10% sucrose with nearly quantitative recovery of activity.

Running gels (5 mm × 6 cm) containing 4% acrylamide were prepared according to Davis (14). No stacking gels were used. Triton X-100 was included in all gels at a final concentration of 0.1% (16). The chamber buffer contained Tris + borate at pH 8.6 (53) instead of the usual Tris-glycine buffer (14). Glycine was omitted from the chamber buffer because it inhibits alkaline phosphatase activity (18). Electrophoresis at 2.5 mA/gel was carried out at room temperature for about 1.5 h until the bromphenol blue tracking dye had nearly reached the end of the gel.

Assay of Alkaline Phosphatase in Gel Slices

The gels were frozen on dry ice, cut into slices 0.8 mm thick, and the alkaline phosphatase activity of each slice was assayed by incubating the slice in 0.25 ml of the assay mixture. The *p*-nitrophenylphosphate concentration was usually reduced to 1 mM for the measurement of pancreatic alkaline phosphatase, and to 5 mM for measurement of the activity of the duodenal enzyme. After incubation at 37°C (up to 10 h), 0.25 ml of 0.2 N NaOH was added to stop the reaction. Recoveries of greater than 75% of the applied activity were routinely observed.

Assay for Carbonic Anhydrase

For assay of carbonic anhydrase, the pancreases were collected in a Beckman microfuge tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and were

homogenized by sonication (55) in the presence of distilled water and *n*-butanol (1:1 by volume). The aqueous phase was collected after centrifugation, and the extraction procedure was repeated three times. The tissue was kept at 0°C during the entire operation. The three aqueous phases were pooled and assayed according to the method described by Maren (34). 1 U represents the amount of activity which decreases the reaction time in the absence of enzyme by one-half.

The carbonic anhydrase assay was not linearly proportional to the enzyme present. For example, a three-fold increase in concentration produced a two-fold increase in activity. Due to the small amount of material available and the low activity, the results are only semiquantitative. With increasing protein concentration of the sample, the reaction time was shortened. This effect, however, is not influenced by the presence of acetazolamide, a specific inhibitor of carbonic anhydrase (4), and is also observed if bovine serum albumin at equivalent protein concentrations is added to controls.

Histochemistry

Detection of alkaline phosphatase was achieved by a modification of Gomori's calcium cobalt method (20). The tissues were fixed for 7 h in 10% Formalin in sodium phosphate buffer, pH 7, and then left overnight at 0°C in 0.88 M sucrose solution containing gum acacia. Frozen sections were cut at −12°C on an International cryostat (International Scientific Instruments, Inc., Mountain View, Calif.) at 10 μm thickness. The sections were mounted on albuminized slides and dried at room temperature for 2 h. They were then transferred to the incubation medium for 2.25 h at 37°C. The incubation medium consisted of 5 ml of 0.01 M *p*-nitrophenylphosphate, 10 ml of 50 mM piperazine-glycylglycine buffer, pH 10, 20 ml of 2% calcium chloride, 1 ml of 5% magnesium sulfate, and 5 ml of H_2O. At the end of the incubation the sections were briefly rinsed in water, treated for 2 min in 2% cobalt nitrate, again rinsed in water, and treated with 1% ammonium sulfide for 2 min. They were then washed in water and mounted in glycerol jelly.

The detection of carbonic anhydrase was made according to the adaptation by Rosen (45, 46) of the Hansson (22) technique. The tissues were fixed at 0°C in 3% glutaraldehyde buffered at pH 7.4 with 0.17 M cacodylate-HCl buffer for 45 min. They were rinsed and stored in saline (0.9% NaCl) until frozen in an International cryostat and cut into 10-μm thick sections. The sections were picked up on Millipore filters (Millipore Corp., Bedford, Mass.) and held in petri dishes on saline-soaked filter paper. The sections were then incubated for 12 min at room temperature in the reaction medium by floating them still attached to their Millipore filter, tissue side uppermost. The reaction medium was prepared by pouring 40 ml of freshly prepared solution II (0.75 g of $NaHCO_3$ in 40 ml of H_2O) into 17 ml of solution I (1 ml of 0.1 M $CoSO_4$, 6 ml of 0.5 M H_2SO_4, 10 ml of 0.066 M KH_2PO_4) contained in a petri dish 9.5 cm in diam. The medium was kept at room temperature for 20 min before use. The specificity of the reaction was tested by incubating other tissue sections simultaneously in similar medium but containing 0.1 mM acetazolamide. The sections were then rinsed three times in 6.7×10^{-4} M potassium phosphate buffer, pH 5.9, before a 3-min incubation in a freshly prepared 0.6 M ammonium sulfide solution. The tissues were then rinsed in saline before being mounted on glass slides with glycerol jelly.

Histological Observations

For light and electron microscope observations, the pancreases were fixed in 2.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) in 0.044 M sodium phosphate buffer at pH 7.4 at room temperature. The tissues were then washed in 0.132 M sodium phosphate buffer and postfixed for 1 h at 0°C in 2% osmium tetroxide in the same buffer. The dehydration and embedding were carried out according to the method described by Luft (32). Thick sections were colored with azure methylene blue and recorded photographically with a Zeiss microscope. Thin sections cut with a diamond knife were collected on 75-mesh grids covered with a Formvar film stabilized with carbon coating. The contrast was increased by treating the section with uranyl acetate at 60°C for 30 min followed by lead citrate (43) and examined with a Philips 300 electron microscope. The sections for light microscope autoradiography were prepared according to the method described by Caro and van Tubergen (8), using an Ilford L-4 emulsion (Ilford Ltd., Essex, England).

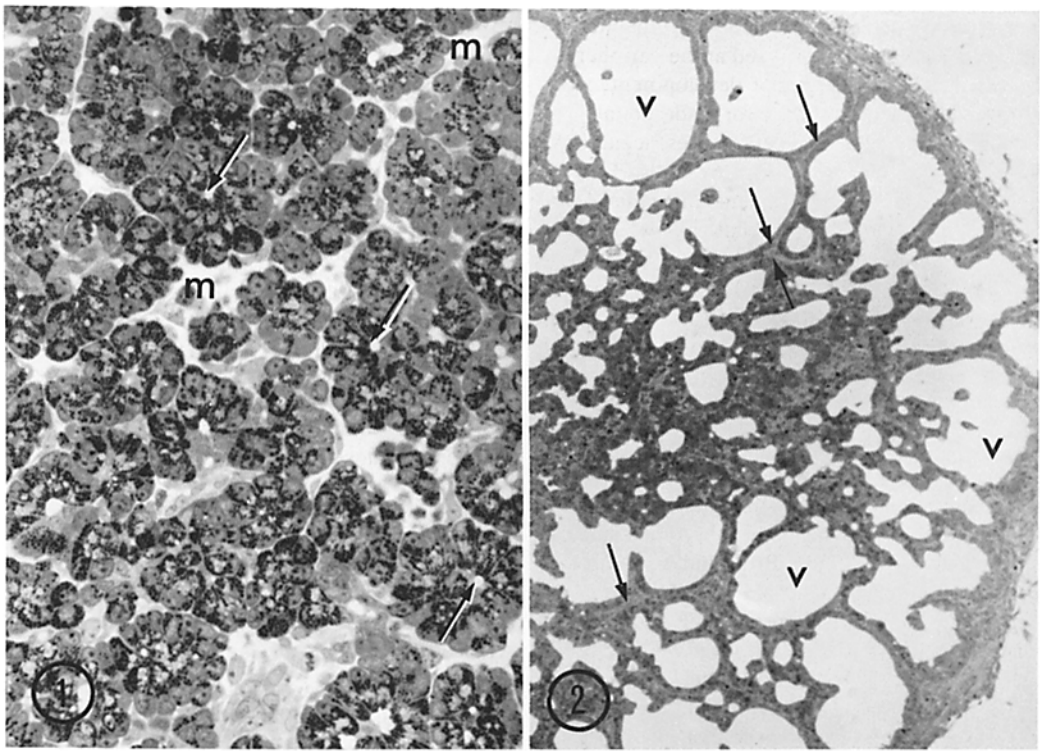
Withdrawal of Fluid from Vacuoles

Glass capillaries with a tip diam of about 1 μm were made with a vertical pipette puller (David Kopf Instruments Model 700C, Tujunga, Calif.). With the use of a micromanipulator, the capillaries were inserted into the vacuoles, and fluid was withdrawn by a combination of capillary action and gentle suction.

RESULTS

Effect of BrdU on Pancreatic Morphology and Fluid Secretion

Exocrine tissue of pancreatic rudiments isolated from 14-day-old embryos, when cultured in the presence of a level of BrdU which selectively inhibits the accumulation of exocrine enzymes, does not develop into normal acinar structures with associated ducts (Fig. 1), but, instead, numerous extracellular fluid-filled vacuoles accumulate throughout the tissue (Fig. 2). The cells lining the vacuoles have the typical exocrine organization: they are disposed in a single layer and are connected by a continuous belt of junctional com-



FIGURES 1 and 2 Morphological appearance of normal and BrdU-treated pancreases in culture. The pancreases were explanted at day 14 and cultured 6 days in the absence (Fig. 1) and presence (Fig. 2) of 20 μM BrdU. The control explant seen in Fig. 1 shows the normal appearance of the exocrine tissue at the end of the differentiation period. The lumen of the exocrine gland (arrows) is small, and most of the pancreatic epithelial cells are acinar cells and contain zymogen granules. The space between the lobules formed by the acini contain the mesenchymal cells (*m*). In contrast, the epithelial cells in the explant treated with BrdU (Fig. 2) surround large vacuoles (*v*) and do not accumulate zymogen granules. The mesenchymal cells (arrows) normally dispersed among the lobules are compressed between the epithelial cells, obliterating the space normally existing between them. Otherwise the BrdU-treated explants are healthy and show no signs of necrosis. Fig. 1, × 320; Fig. 2, × 80.

plexes limiting their apical surface. The basal side is encompassed by the basal lamina which separates these cells from the mesenchymal cells. The cells in BrdU-treated rudiments (Fig. 3) resemble the pancreatic duct cells of the normal pancreas of corresponding age (Fig. 4): they are cuboidal in contrast to the pyramidal shape of the acinar cell, are devoid of zymogen granules, and most of them lack the extensively developed rough endoplasmic reticulum typical of acinar cells (Fig. 4). The size of the fluid-filled vacuoles depends on the concentration and the length of the exposure to BrdU; the largest vacuoles are present at the periphery (Fig. 1). The vacuolization starts to develop 2–3 days after the addition of the BrdU and progressively increases during the following days. After 5 or 6 days the largest vacuoles fuse and their resulting size allows withdrawal of the fluid as described in Materials and Methods. The fluid withdrawn from the vacuoles is replaced in less than 24 h.

The formation of the vacuoles is correlated with the incorporation of the BrdU into DNA. When pancreases grown in continuous presence of 20 μM BrdU are labeled with [^{3}H]BrdU for a few hours, a large proportion of the cells incorporate the label into the nuclei (Fig. 5). The proportion of nuclei incorporating the [^{3}H]BrdU label is similar to the proportion of nuclei incorporating [^{3}H]thymidine in the control cultures during the same period of labeling. Concomitant with the end of the differentiation period there is a sharp de-

crease in cell proliferation and the remaining dividing cells are mostly localized at the periphery of the tissue. At this period of development, when BrdU is added to the culture for 4 additional days, vacuoles lined by ductlike cells are restricted to the periphery of the culture (Fig. 6). Pulse labeling with [^{3}H]BrdU and subsequent autoradiography demonstrates that the epithelial cells which have incorporated the thymidine analogue are located around these vacuoles (Fig. 6). We conclude that the effects of BrdU are most likely a result of this incorporation into cellular DNA.

Opposite Effects of BrdU on Alkaline Phosphatase and Amylase Activities

In contrast to its inhibition of accumulation of exocrine proteins, BrdU stimulates the alkaline phosphatase activity of the embryonic pancreas cultured in vitro. This BrdU-induced alkaline phosphatase has a high pH optimum ($\geq$10.5) (Fig. 7) and increases in proportion to the BrdU concentration in the medium (Fig. 8). The previously reported (55) inhibitory effect of BrdU on specific exocrine protein accumulation in this system was evidenced by the progressively decreased levels of amylase as the concentration of BrdU was raised. The small increase detected in the specific activities of acid phosphatase and catalase are probably related to the small depression in the amount of protein per rudiment. This inhibition of protein accumulation is mostly due to the inhibition of accumulation of specific exocrine proteins, which normally comprise about 40% of the total proteins of the fully developed pancreas under these culture conditions.[1]

Assay of pooled BrdU-treated and untreated rudiments showed that the increase in alkaline phosphatase activity caused by BrdU was not due to a diffusible activator. This conclusion was supported by the fact that greater than 75% of the BrdU-stimulated alkaline phosphatase activity could be recovered in gel slices after polyacrylamide gel electrophoresis.

The total alkaline phosphatase activity per untreated rudiment increased slightly during the culture period, while the protein-based specific activity did not. In the BrdU-treated rudiments there was a lag of approx. 2 days before a steady increase in alkaline phosphatase activity began.

Continuous exposure to BrdU is not required for the stimulation of alkaline phosphatase. As shown in Table I, 1–3 days' exposure of 14-day rudiments to BrdU at the beginning of the culture period caused a proportional increased accumulation of alkaline phosphatase after 6 days. BrdU treatment during only the first 3 days was as effective as a continuous exposure for 6 days, in agreement with the 2–3-day lag observed, while shorter exposures had lesser effects. These observations are consistent with our contention that the BrdU effect is a result of incorporation of this analogue into the DNA. Older and more fully differentiated rudiments were also sensitive to BrdU. As shown in Table II, rudiments exposed to BrdU late in the culture period also exhibited an increase in alkaline phosphatase activity, provided the period of exposure was sufficient. In all of these experiments, the extent of inhibition of amylase accumulation mirrored the stimulation of alkaline phosphatase (Table I).

The degree of vacuolization of the BrdU-treated tissue also paralleled the accumulation of alkaline phosphatase activity. This correlation held when the pancreas was exposed to BrdU for only a few days or when exposure was begun later in the culture period.

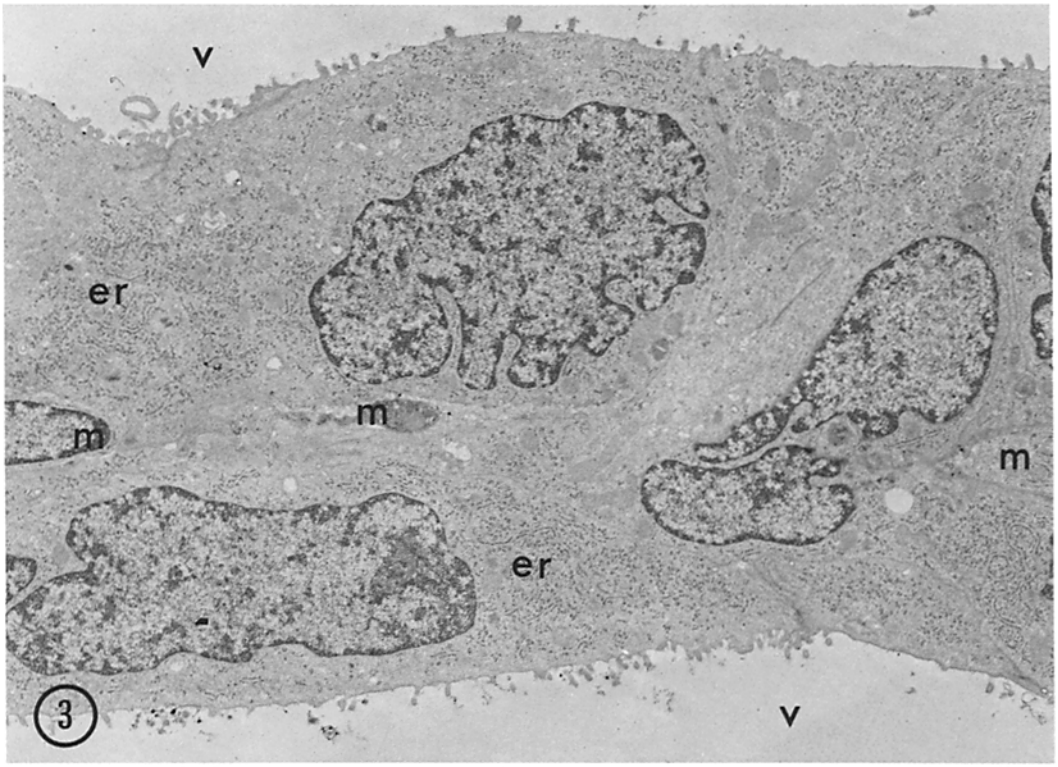
Effect of BrdU on ATPase Activities

The fluid secretory activity of the adult pancreas might depend on the ouabain-sensitive (Na + K) ATPase (44) or on a bicarbonate-stimulated ATPase activity (52), both of which are maximally active at about neutral pH. It has been suggested that some alkaline phosphatase activities may be side reactions of such ATPases (47). However, the increase in pancreatic alkaline phosphatase activity was not accompanied by an increase in either of these ATPase activities, and no Ca^{++}-stimulated ATPase could be detected. This result is consistent with the finding that the large stimulation of liver alkaline phosphatase by a variety of agents, e.g., hydrocortisone, was not accompanied by an increase of neutral Mg-ATPase activity (40). Thus, there is no apparent relationship between the alkaline phosphatase activity and these ATPases.

Comparison of Alkaline Phosphatase Activities of Pancreas and Duodenum

The alkaline phosphatase activities of various tissues may be discriminated on the basis of relative inhibitions by L-phenylalanine (19) and urea

[1] Rall, L. B., S. Githens, R. L. Pictet, and W. J. Rutter. Manuscript in preparation.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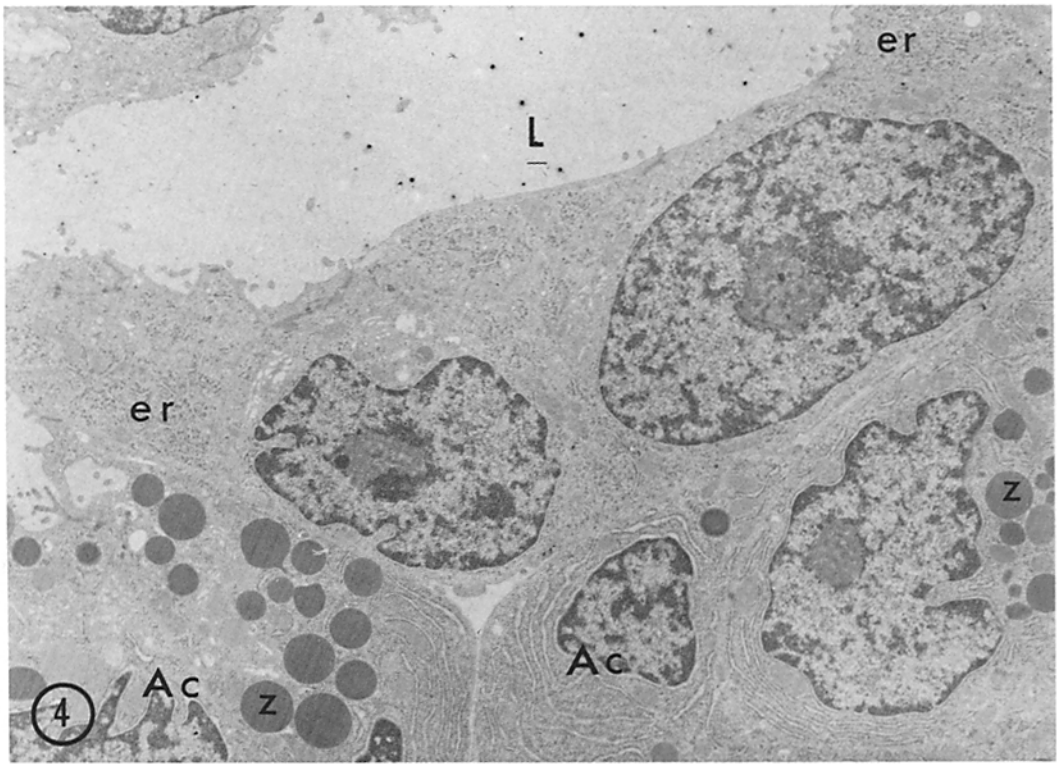
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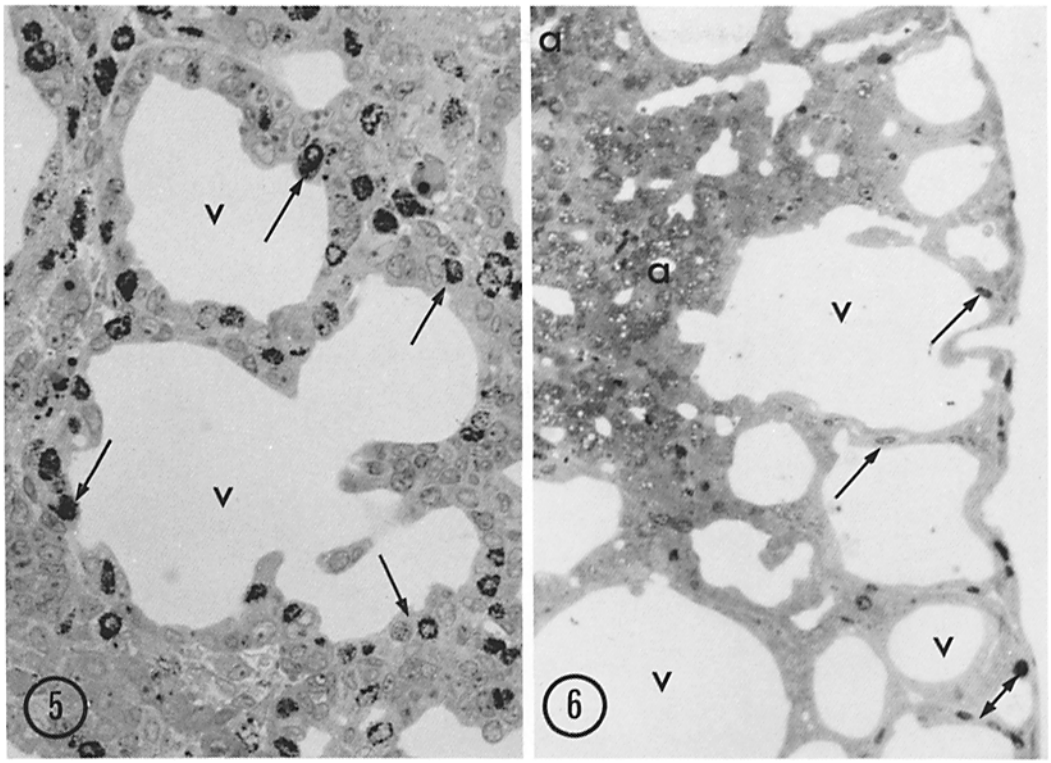


FIGURE 5 Many cells incorporate BrdU into nuclei. 14-day pancreases were explanted and cultured for 3 days in the presence of 20 μM BrdU. 10 μCi of [^{3}H]BrdU were added for 6 h, then explants were harvested and processed for light microscope autoradiography. Even after a short period of labeling, numerous labeled epithelial cells border the forming vacuoles (arrows). This sign of active DNA synthesis correlates with the DNA increase occurring during the entire culture period (5 or 6 days) (55). Parallel experiments show that roughly the same number of cells incorporate the [^{3}H]DNA precursor in both tissues, control or BrdU-treated, if [^{3}H]thymidine or [^{3}H]BrdU is added to the culture medium. × 350.

FIGURE 6 Pancreases explanted at day 14 of gestation were cultured for 4 days in regular medium, and the following 4 days in the presence of 20 μM BrdU. 6 h before harvesting, 10 μCi of [^{3}H]BrdU was added. They were then fixed and processed for light microscope autoradiography. The vacuoles surrounded by cells which mostly do not contain zymogen granules are localized at the periphery of the tissue culture. The labeled cells are also restricted mostly to the periphery of the culture. This is in agreement with the concept that the vacuole formation is a consequence of the incorporation of BrdU in the nuclei of the epithelial cells. At 22 days, only a few cells divide; thus, only a few cells are labeled. Differentiated acinar cells (*a*). × 90.

FIGURES 3 and 4 Ultrastructural appearance of BrdU-treated and normal pancreatic cells. Pancreases explanted at day 14 were grown for 6 days in the presence (Fig. 3) and absence (Fig. 4) of 20 μM BrdU. Fig. 3 shows the cells of the walls of two contiguous BrdU-induced vacuoles. The cells surrounding the vacuoles look like the duct cells seen in normal pancreas of the same age (cf. Fig. 4). There is little development of the rough endoplasmic reticulum (*er*). The cells are more cuboidal than the pyramidal shape of the acinar cells. The apical surface facing the vacuoles (*v*), which corresponds to the lumen of duct and acini in the normal pancreas, shows the characteristic short microvilli also seen in duct cells. Some mesenchymal cells (*m*) are present between the two layers of epithelial cells. The normal duct cells surround a small lumen (*L*). These cells are similar to in vivo fetal or newborn pancreatic duct cells. In contrast with the acinar cells (*Ac*) containing zymogen granules (*z*), the duct cells have little rough endoplasmic reticulum (*er*) which becomes even less abundant in adult duct cells. Fig. 3 and 4, × 5500.

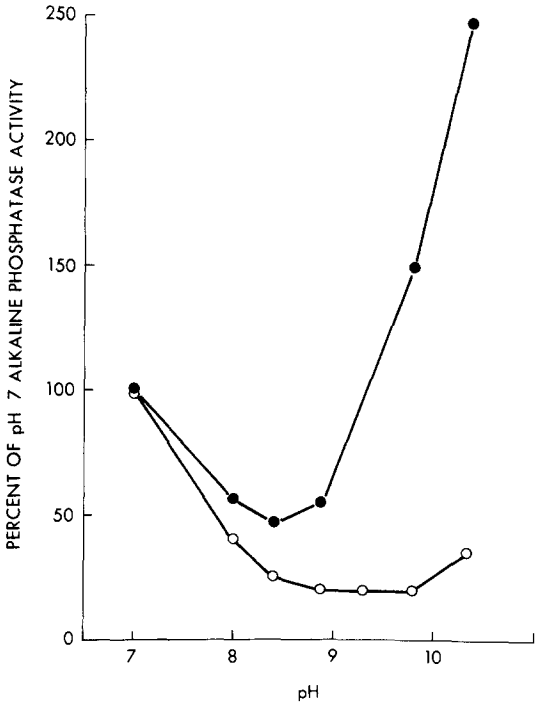


FIGURE 7 pH profile of alkaline phosphatase activity in pancreatic rudiments grown in the presence and absence of BrdU. Pancreatic rudiments were grown from day 14 to day 19 in the presence and absence of 20 μM BrdU and were assayed for alkaline phosphatase activity from pH 7 to 10.5 as described in the Materials and Methods section. The results are expressed in percent of pH 7 activity.

The activities were, at pH 7: for the control, 12.5 mU/mg protein; and for the experimental, 21.0 mU/mg protein. This difference in specific activity is due to the absence of exocrine products, which form 40% of the total cell proteins. (●) BrdU; (○) control.

(7), relative activities against different substrates such as *p*-nitrophenylphosphate and β-glycerophosphate (61), and electrophoretic mobility (29). We have characterized the BrdU-stimulated alkaline phosphatase activity of the pancreas by comparing it according to the above criteria with various pancreatic and duodenal alkaline phosphatase activities. The duodenal tissues were included in these studies since the duodenum characteristically exhibits a marked increase in alkaline phosphatase activity during development (37), and the pancreas originates from the portion of the gut that will become duodenum. Therefore the BrdU-treated pancreas might exhibit a duodenal-like alkaline phosphatase activity.

As shown in Table III, the specific alkaline phosphatase activities of adult pancreatic ducts and islets were much higher than that of the adult pancreas as a whole. Thus, the activity of this enzyme in the pancreas is due primarily to the activity of its duct and islet (21, 36) components. The alkaline phosphatase specific activity of embryonic pancreases cultured in the presence of BrdU resembled the specific activities found in the isolated islets and ducts. However, there is no increase in the islet cell population or in the insulin specific activity in BrdU-treated rudiments.[2] Moreover, if BrdU is added to day-13 pancreases, B-cell development as well as insulin accumulation are inhibited, whereas the alkaline phosphatase activity increases as usual. Thus, we conclude that the increased alkaline phosphatase is not derived from an increased number of the endocrine cells. The increased alkaline phosphatase activity is associated with epithelial cells and not mesenchymal cells since a pure pancreatic epithelial cell population freed of mesenchymal cells (as described in Materials and Methods) shows an increase in alkaline phosphatase specific activity similar to that of the intact rudiment when cultured in the presence of BrdU (Table III).

The embryonic duodenum exhibited a moderate alkaline phosphatase activity, which increased about four-fold during in vitro culture, considerably less than the cortisone-induced increase observed in vivo (37). BrdU exerted a slight effect if any on the alkaline phosphatase activity in the duodenum. The adult duodenum exhibited its characteristic high alkaline phosphatase activity.

Gel electrophoretic analysis revealed a single major peak of alkaline phosphatase activity with an R_f value of 0.31–0.33 in the following tissues: adult pancreas, adult pancreatic islets and ducts, embryonic pancreas grown in vitro from day-14 to day-20 in the presence and absence of 20 μM BrdU, and embryonic duodenum grown in vitro for the same period of time. The adult pancreas always contained a minor alkaline phosphatase activity (R_f = 0.52), which was never seen in the other tissues examined. In contrast to the above, the adult duodenal alkaline phosphatase exhibited its characteristic mobility of R_f = 0.22 (23, 57).

The substrate specificity (61) as well as the effects of the inhibitors urea (7) and L-phenylalanine (19) were determined. As shown in Table III, the alkaline phosphatase activities of all of the

[2] Rall, L. B., R. L. Pictet, and W. J. Rutter. Manuscript in preparation.

pancreatic tissues resembled each other by these criteria. The duodenal enzyme on day-14 of gestation resembled the pancreatic activities, but after 7 days of culture the activity exhibited the characteristics of the 20-day in vivo embryonic and adult duodenal activities. BrdU only partially blocked this shift in enzymatic properties. These results show that the activity which accumulates in the pancreas in the presence of BrdU is identical, by a variety of criteria, to that normally found in the pancreas and is not the same enzyme which normally accumulates in the mature duodenum.

Glucocorticoids have no Effect on Pancreatic Alkaline Phosphatase or Fluid Secretion

Alkaline phosphatase is induced by dexamethasone in several cell types (13) including duodenum (38). In contrast, the alkaline phosphatase in the embryonic pancreas was not stimulated by the addition of hydrocortisone (10 μM) or dexamethasone (1 μM) to the culture medium. These glucocorticoids did, however, cause a significant increase in amylase accumulation.[1]

Effect of BrdU on Carbonic Anhydrase Activity

After 6 days in culture in the presence of 20 μM BrdU, the pancreases explanted at day-14 of development have about 50 U of carbonic anhydrase activity per mg DNA. This activity is entirely suppressed by addition of 0.1 mM acetazolamide, a specific inhibitor of carbonic anhydrase. In pancreases which were not cultured in the presence of BrdU, no acetazolamide-suppressible activity was detected.

Histochemical Detection of Alkaline Phosphatase and Carbonic Anhydrase

The variation of alkaline phosphatase and carbonic anhydrase activities between normal and BrdU-treated pancreatic rudiments correlates with increases in these activities detected by histochemical methods. Fig. 9 shows the distribution of alkaline phosphatase activity in cultures grown in the absence of BrdU. The positive reaction is localized in some undifferentiated exocrine (duct) cells and more weakly in endocrine A cells. In pancreases grown in the presence of BrdU, there is a large amount of alkaline phosphatase activity in the cells lining the vacuoles, although the intensity of the reaction varies from cell to cell (Figs. 10 and 11). The cells located between the vacuoles show an even stonger positive reaction. These cells, as seen with the electron microscope, consist of mesenchymal and endocrine, mostly B, cells. (Endocrine cells accumulate normally when BrdU is added after day 13.)[2]

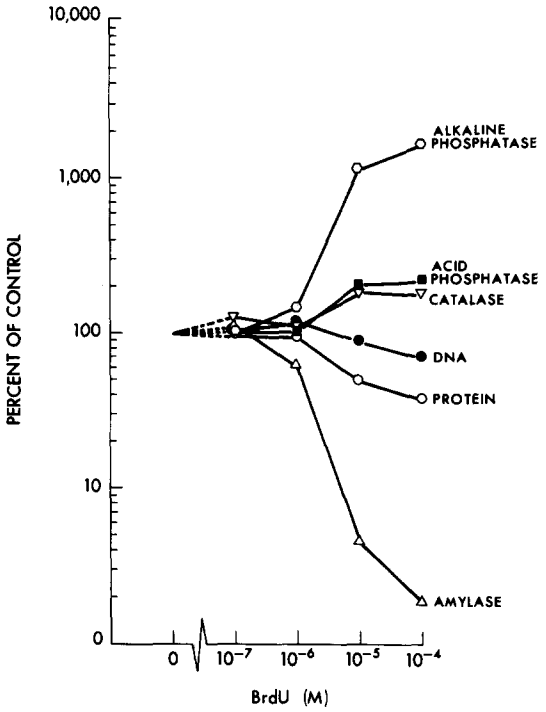


FIGURE 8 Inverse effect of BrdU on the accumulation of amylase and alkaline phosphatase activities in developing pancreatic rudiments. Pancreatic rudiments cultured from day 14 to day 19 in varying concentrations of BrdU were harvested and assayed for the indicated substances. Each activity was expressed as a percentage of the value found in the control sample, which contained 85 μg of protein and 4.35 μg of DNA per rudiment and the following enzymatic activities: 1.54 mU of alkaline phosphatase, 32.5 mU of acid phosphatase, 10.95 U of catalase and 13.7 U of amylase per mg protein. The small increase in acid phosphatase and catalase specific activity in the presence of the highest BrdU concentration is due to the decrease in the exocrine enzymes.

The distribution of the alkaline phosphatase reaction products is strongly dependent upon the pH at which the reaction is carried out. Only at pH 10 is there a clear positive reaction in the cells lining the vacuoles. Between pH 8.5 and 9.5, it is restricted to the cells located between the vacuoles,

TABLE I

Reciprocal Effects of BrdU on Amylase and Alkaline Phosphatase of the Developing Pancreas

BrdU treatment	Amylase	Alkaline phosphatase
days	*U/mg protein*	*mU/mg protein*
None	23.0	1.4
19–20	16.6	1.6
18–20	8.8	2.1
17–20	11.8	4.1
14–15	4.4	8.2
14–16	<1	14.4
14–17	<1	22.6
14–20	<1	22.9

Pancreatic rudiments were placed in culture on day 14, exposed to 20 μM BrdU for the indicated number of days, and harvested on day 20 for assay.

TABLE II

Increase in Alkaline Phosphatase Activity is a Function of the Length of the Exposure to BrdU

Exp	Day of initial exposure to BrdU	Alkaline phosphatase activity (Increase over a 2-day period)	
		day 18–20	day 20–22
		mU/mg protein	
I	14	25	–
	15	25	–
	16	12	–
	17	6.5	–
	none	0	–
II	14	–	9
	17	–	12.6
	18	–	6.5
	19	–	2.5
	none	–	0.5

Pancreatic rudiments were placed in culture on day 14. 20 μM BrdU was added on the indicated day, and the rudiments were harvested on either days 18 and 20 (exp I) or 20 and 22 (exp II) and assayed for alkaline phosphatase. The numbers represent the increase in activity from days 18 to 20 and 20 to 22, respectively. The magnitude of the effect of BrdU is more dependent on the length of exposure than on the stage of development reached when BrdU is added. However, when added at day 14 a plateau has already been reached by day 20, and no further increase in activity is observed.

with very few vacuole cells showing a weak positive reaction.

The pH optimum of the alkaline phosphatase reaction in the epithelial cells of the rudiment suggests that the alkaline phosphatase stimulated by BrdU (Fig. 7) is localized in the cells lining the vacuoles. The strong reaction seen at a lower pH in the interstitial cells is apparently due to other phosphatases which are present (Fig. 7), and which are also stimulated to some extent by BrdU. It is also possible that the relative prominence of the phosphatase activity in the interstitial cells was due to the assay conditions of the histochemical technique.

The carbonic anhydrase in BrdU-treated pancreases is located in the cells bordering the vacuoles. Some of the scattered positive cells may also be cells lining vacuoles to which the plane of section is tangential (Fig. 12). The reaction was totally inhibited by acetazolamide (Fig. 13). In rudiments cultured in the absence of BrdU, only a few cells, probably corresponding to duct cells, show a positive reaction (Fig. 14).

DISCUSSION

The differentiation of the rat exocrine pancreas occurs in two distinct stages (24, 41, 42, 50). The first differentiative transition (10–11 days) results in the protodifferentiative state which is characterized by low but significant levels of specific exocrine products. During this period (about 2 days), there is extensive morphogenesis and the formation of acinar structures. However, there is no ultrastructural means for differentiating between the acinar and duct cells. The second transition (14–16 days of gestation) involves a dramatic rise in the synthetic rate of the exocrine enzymes leading to differentiated (adult) levels of those enzymes (24). This rapid accumulation is correlated with cytodifferentiation of the large majority of the exocrine cells into acinar cells which form the tips of the exocrine digitations. Already in the late fetus (20 days) as in the adult, these acinar cells are clearly the major cell type of the differentiated pancreas; the remaining exocrine cells do not contain zymogen granules (42). These cells comprise the so-called duct cell population: one function of the duct is to conduct the acinar cell products to the gut. At least some of these cells also secrete the alkaline bicarbonate-rich buffer produced by the exocrine pancreas.

During the protodifferentiated stage the presence of BrdU in the culture medium for an appropriate period leads to the accumulation of a majority of exocrine cells which, instead of being acinar, resemble duct cells by morphological, functional, and biochemical criteria. The identification of the duct cells after BrdU treatment is also based on the simultaneous accumulation of increased levels

of alkaline phosphatase and carbonic anhydrase. High levels of alkaline phosphatase are associated with tissues active in fluid transport (23), and carbonic anhydrase is thought to have a role in the formation of the bicarbonate-rich pancreatic fluid that is secreted by the pancreatic ducts (35). The BrdU-stimulated alkaline phosphatase activity resembled the alkaline phosphatase of control pancreatic tissues by all criteria applied.

The histochemical analysis of alkaline phosphatase distribution after BrdU treatment was complicated by the simultaneous appearance of phosphatase activities in both interstitial cells and the cells lining vacuoles. In the normal embryonic as in the adult pancreas, alkaline phosphatase is present in blood vessels, islets and ducts (15, 21, 36) and in mesenchymal cells associated with large ducts (15). Blood vessels do not develop in pancreases grown in vitro (42). Alkaline phosphatase is not detectable in acinar cells (21). Treatment by BrdU of pancreatic rudiments before 14 days of gestation blocks both the endocrine B-cell and acinar differentiation, whereas at 14 days or later the accumulation of exocrine enzymes is inhibited, and there is little if any effect on insulin content. In both cases the effect on alkaline phosphatase is the same. Since, as seen with the electron microscope, there is no change in islet A and B cell number in BrdU-treated pancreases, the enhancement in alkaline phosphatase does not originate from an enlarged islet cell population. Since BrdU stimulated the biochemically assayed alkaline phosphatase activity of a pure epithelial cell population to the same extent that it stimulated the alkaline phosphatase activity of the entire rudiment, we conclude that mesenchymal cells are also not involved in a major way in the response to BrdU. Histochemical analysis showed that the epithelial alkaline phosphatase exhibited a higher pH optimum than the activity present in mesenchymal cells. The mesenchymal activity detected in the histochemical analysis was then probably due to the enzyme with a lower pH optimum. This enzyme may also be more resistant to fixation. This contention is supported by the fact that at lower pH (8–8.5) where only mesenchymal cells are positive there is little (two-fold or less) stimulation of assayable activity over the control level.

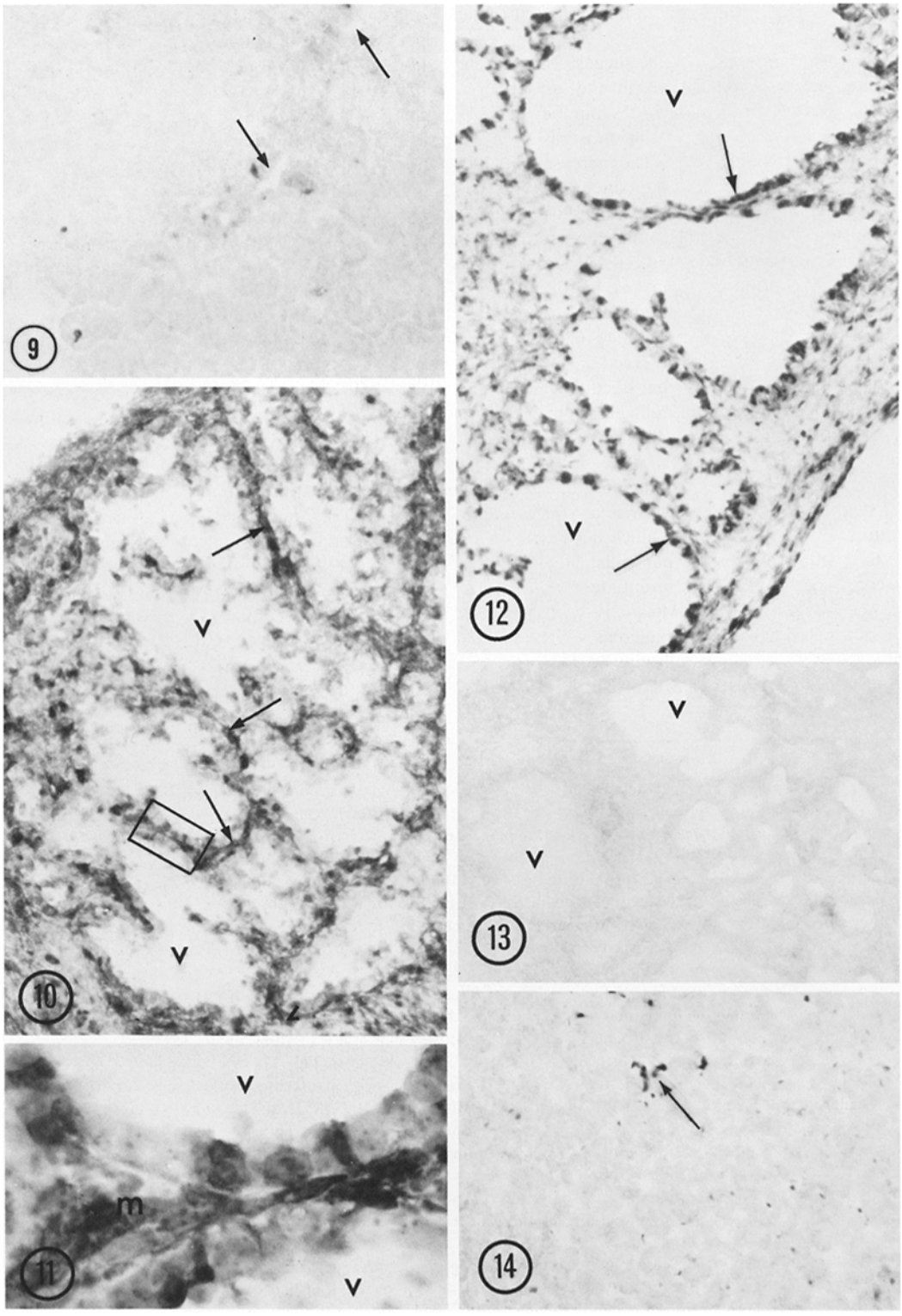
The studies with carbonic anhydrase strongly support the conclusion that BrdU causes an increase of duct cells over the proportion existing in rudiments of equivalent age grown in the absence of BrdU. There was little or no activity in control cultures and an obvious increase in carbonic anhydrase activity in the BrdU-treated tissue. Furthermore, the histochemical procedure showed re-

TABLE III

Characteristics of Alkaline Phosphatase Activities of Pancreas and Duodenum at Different Developmental Stages

Tissue	Gestational age	In vitro culture	Exposure to BrdU	Alkaline phosphatase	Inhibition		Substrate specificity	R_f
					Phenylalanine	Urea		
	days	*days*		*mU/mg protein*	*%*			
Pancreas	14	0	–	4.6	10	79	0.16	–
"	20	0	–	1.3	7	64	–	–
"	21	7	–	1.8	10	79	0.13	0.31
"	21	7	+	31.1	14	87	0.19	0.33
Pancreatic epithelia	20	8	–	2.6	–	–	–	–
"	20	8	+	38.9	–	–	–	
Pancreas	Adult	0	–	1.6	14	73	<0.10	0.32, 0.52
Pancreatic ducts	Adult	0	–	56.0	9	89	0.23	0.31
Pancreatic islets	Adult	0	–	22.0	15	79	–	0.31
Duodenum	14	0	–	3.7	0	71	0.12	–
"	20	0	–	364	57	0	–	–
"	20	6	–	23.5	44	19	0.86	0.31
"	20	6	+	30.6	35	35	–	–
"	Adult	0	–	950	47	38	0.56	0.22

Embryonic tissues were isolated for immediate assay or were cultured for the indicated periods in the presence or absence of 20 μM BrdU. Adult tissues were obtained as described in Materials and Methods. Inhibition was measured in the presence of 15 mM phenylalanine or 3 M urea in the assay medium. Substrate specificity represents the activity with β-glycerophosphate as a substrate divided by activity with p-nitrophenylphosphate. R_f represents the mobility of enzymatic peak relative to the mobility of the tracking dye.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sponse mostly if not solely in the epithelial (ductlike) cells.

Our experimental results suggest that in the embryonic pancreas grown in the presence of BrdU there is an increase in the number of functioning duct cells. The conclusion is based on the following observations: (*a*) The increased alkaline phosphatase activity is of the pancreatic type, not the duodenal type; (*b*) the alkaline phosphatase is not due to endocrine cells and is at most modestly contributed by mesenchymal cells; (*c*) histochemical analysis, although unfavorable for duct cell alkaline phosphatase, nevertheless demonstrates activity in the epithelial ductlike cells; (*d*) the increased carbonic anhydrase activity is found almost exclusively in epithelial ductlike cells lining the vacuoles; and (*e*) the cells grown in the presence of BrdU, like duct cells in vivo, are actively involved in fluid transport. At least two mechanisms may account for this increase in duct cells. First, the pancreas may comprise a population of pluripotent precursor cells which normally differentiate into duct and acinar cells. BrdU causes these precursor cells to differentiate into duct cells rather than acinar cells. Alternatively, the protodifferentiated cells of the pancreas which are the precursors of the acinar cells may be duct cells. By blocking this transition, BrdU "causes" an accumulation of duct cells. This second mechanism is perhaps favored by the observation that the addition of cyclic AMP derivatives (cyclic AMP is the intracellular mediator of secretin action in duct cells) to the medium of cultured 12-day epithelia leads to the formation of vacuoles and accumulation of fluid in less than 24 h (17).

A similar stimulation by BrdU of alkaline phosphatase activity has been previously reported in a hybrid cell line derived from mouse mammary carcinoma and Chinese hamster lung cells (25–27). The kinetics and magnitude of the effect correspond generally with our results; in this cell line there is an initial 24-h lag and a maximal increase of about 24-fold in specific activity at 16 μM BrdU after 6 days in culture. In further analogy with our results, the hybrid line's ability to synthesize hyaluronic acid (a "differentiated" function) was inhibited by BrdU proportionally to the degree of stimulation of alkaline phosphatase activity (25). The induction of alkaline phosphatase appears to be a property of the mammary cell component of the hybrid since BrdU induces alkaline phosphatase in parental mammary cells but not in lung cells (27). From the above, it seems possible that BrdU will exert an effect on mammary gland differentiation entirely analogous to its effect on the pancreas. The mammary tumor cells

FIGURE 9–14 Histochemical reaction for alkaline phosphatase and carbonic anhydrase. Figs. 9–11: Alkaline phosphatase activity in BrdU-treated pancreatic rudiments. The pancreases were explanted on day 14 of gestation and cultured for 5 days in the presence and absence of 20 μM BrdU. They were then harvested and processed for histochemical detection of alkaline phosphatase activity as described in Materials and Methods. The normal pancreases (Fig. 9) show little alkaline phosphatase activity. Its localization is restricted to clusters of cells corresponding to endocrine cells, as observed with the electron microscope, and cells localized along lumens (arrows) corresponding to duct cells. In contrast, the BrdU-treated tissues (Fig. 10) show a strong reaction which is present in both interstitial cells which include mesenchymal cells (arrows) and epithelial cells which line the vacuoles. As better seen at high magnification in Fig. 11 (boxed area of Fig. 10), the intensity of the reaction varies among the epithelial cells. The stronger reaction seen in the interstitial cells may indicate that the histochemical activity is not quantitative. This is supported by the fact that pancreases deprived of mesenchymal cells show the same increase in alkaline phosphatase specific activity (see text). Figs. 9 and 10, × 110; Fig. 11, × 620. Figs. 12–14: Carbonic anhydrase activity in BrdU-treated pancreatic rudiments. The pancreases explanted on day 14 were cultured in the presence (Figs. 12, 13) and absence (Fig. 14) of 20 μM BrdU. They were harvested and processed for carbonic anhydrase reaction as described in the Materials and Methods section. The reaction is present in most of the epithelial cells bordering the vacuoles (Fig. 12). The specificity of the reaction is demonstrated by the lack of reaction product when incubation is carried out in the presence of 0.1 mM acetazolamide as seen in Fig. 13. In the control rudiments not treated with BrdU, only a few cells (arrows), probably duct cells, show some reaction product. The acinar cells are deprived of activity. Figs. 12–14, × 110.

To allow comparison, the sections were cut, photographed and printed in the same conditions. However, in order to see the tissue, the control samples (Figs. 9, 13, 14) had to be exposed longer, artificially increasing the density of the unreacting tissue and the label in the few positive cells.

may have been derived from a precursor cell which could be "induced" by BrdU to become a mammary duct cell.

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