DNA SYNTHESIS, MITOSIS, AND DIFFERENTIATION IN PANCREATIC ACINAR CELLS *IN VITRO*

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ABSTRACT

Pieces of mouse embryonic pancreatic epithelium cultured in an inductive situation *in vitro*, or when examined at critical times *in vivo*, show a gradient of zymogen granule accumulation. Cells located internally in explants, or in central acini *in vivo*, show this overt differentiation first. As the epithelia age, the more peripheral cell population proceeds in a similar differentiation. Observations of autoradiograms of H^3-thymidine–labeled tissues indicate that the first cells which cease incorporating the DNA-precursor are in the central regions that differentiate first. In older explants, thymidine incorporation is largely restricted to the periphery of the tissue as zymogen appears in the internal cells. Evidence suggests that cells or nuclei which have replicated DNA move inward before dividing. Some daughter cells apparently return peripherad to divide again, whereas others remain centrally where they undergo differentiation. During at least the first 24 hours of these maturational changes, mesenchyme has a stimulatory effect upon epithelial thymidine-incorporation frequencies. The presence of a post-DNA-synthetic population is seen in the form of a group of non-labeling central cells that remains intact in the midst of a labeled epithelium for as long as 48 hours *in vitro* (from 72 to 120 hours). If explants are treated with 5-bromodeoxyuridine for any 24-hour segment of the 0 to 72-hour period, before the non-incorporating population arises, no subsequent overt zymogen formation occurs. If explants are treated continuously from 72 to 120 hours, on the other hand, zymogen still forms in some internal cells. Presumably, this differentiation is limited to the postmitotic population as revealed in the thymidine autoradiograms.

The relation of embryonic induction to the gradual stabilization of cells in a non-dividing, differentiated state can only be elucidated when interacting tissues have been precisely defined in both biological and biochemical terms. Developmental studies (11) and initial electron microscopic observations (16) on the mouse pancreas developing *in vitro* have begun to define certain parameters of this inductive system. The present report records data on the patterns of cell division and exocrine differentiation of the pancreatic epithelium, together with information on the time of cessation of mitosis in differentiating cells.

Many discussions are available on the exclusivity of differentiation and division in embryonic cells (2, 6, 12, 35, 36), but it is only recently that critical evidence has been forthcoming on the precise stages in the cytodifferentiative sequence beyond which DNA is no longer replicated (21, 27, 38). Particularly pertinent is the work of Stockdale and Holtzer (32) who used tritiated thymidine to demonstrate that differentiating chick muscle cells cease incorporating the DNA-precursor before myoblasts fuse to form multinucleate myotubes or synthesize myosin. In a preliminary report (33) the same group treated developing muscle cells

with 5-bromodeoxyuridine (BUDR), a compound that can be incorporated into DNA in place of thymidine (39). They note that postmitotic cells are insensitive to BUDR treatment and differentiate normally. Conversely, the less mature, dividing cells are susceptible to the thymidine analogue and fail to form myotubes or normal quantities of myosin. Presumably, this results from BUDR incorporation into DNA and the subsequent ineffectiveness of the abnormal DNA in supporting myogenesis (*i.e.*, cell fusion, specific protein synthesis).

The results of the experiments to be reported are in general conformity with those cited above on muscle (32), bone (38), and antibody-producing cells (21). The embryonic pancreatic epithelium *in vitro* is peculiarly favorable for this kind of study because it segregates into subpopulations: DNA-synthesizing, mitotic, and non-dividing but differentiating. This report deals with: (*a*) a descriptive study of the thymidine-incorporating, mitotic, and zymogen-producing populations both *in vitro* and *in vivo*; (*b*) experiments on the dynamics of the mitotic population and on the relation of mesenchyme to epithelial mitosis; and (*c*) experiments demonstrating the presence of a postmitotic, BUDR-insensitive group of differentiating exocrine cells.

MATERIALS AND METHODS

The procedures employed in obtaining embryonic mouse pancreas tissues (BALB/C females mated with C3H males) and the techniques for their culture have been described in detail (11, 16). The standard preparation in these experiments consisted of salivary gland mesenchyme, from a 13-day mouse embryo, placed on the upper surface of a membrane filter, with a quarter piece of 11-day embryonic mouse pancreatic epithelium clotted directly beneath the mesenchyme. For all subsequent manipulations, only those preparations were used which displayed a rounded, compact epithelial mass during the culture period.

For labeling of tissues, tritium-labeled thymidine (H^3TDR) (New England Nuclear, Boston; specific activity 3.15 and 6.59 curies per millimole) in Tyrode's solution was mixed, one part to 10 parts with the same nutrient medium that was used for culturing. After varying periods of culture, the filter assembly and attached tissues were transferred to another culture dish containing the pre-warmed labeling solution with H^3TDR at 10 μc per milliliter. The label dish was then reincubated at 38°C in a humidified, 5 per cent CO_2 atmosphere for 1 hour. Some assemblies were then fixed in Bouin's fluid with 0.7 per cent NaCl, or in Carnoy's. Alternatively, other assemblies were washed in fresh nutrient medium for about 5 minutes before being reincubated in additional medium. Total numbers of explants submitted to these various procedures are summarized in Table I.

For comparison with cultured material, pieces of freshly isolated, intact pancreas were labeled after being removed from 10 to 18-day embryos and from adult mice. After excision, tissues were transferred for labeling to the inverted cup of a filter assembly, and then were handled precisely as described above for cultured explants.

Following paraffin embedding and sectioning at 5 μ, standard autoradiograms were prepared, using AR-10 stripping film and employing techniques already reported (19, 37). Sections of each explant were exposed for 3 to 5 and 10 to 15 days, and, in addition, several sections on separate microscope slides were stained with hematoxylin and eosin, or with the Feulgen reagents (24).

The nature of the bound H^3TDR was examined by treating Carnoy's-fixed sections of labeled cultures with deoxyribonuclease (Worthington, Freehold, New Jersey; 1 mg/ml in veronal buffer, 0.028 M, at pH 7.5, and in the presence of $MgCl_2$, 0.0025 M) for 3 hours at 37°C (24). Duplicate sections were incubated in the same buffer system minus DNase. Following washing, film was applied to the slides and they were exposed for 13 days. Upon examination, controls revealed normal grain-densities and distributions for typical 5-day explants. In contrast, the DNase treatment removed all but an exceedingly small amount of the grain-producing radioactive substance. A few grains were still visible in scattered cells, sometimes over cytoplasmic regions. These results support the premise that the H^3TDR being administered is retained in fixable form largely in nuclear, DNase-sensitive material. Taken together with the large body of literature on thymidine incorporation (as 34), these results seem to justify the assumption that thymidine is being bound largely into DNA.

Data on approximate cell generation times were gathered in 3 experiments (15 to 18 cultures each) in which 24- or 72-hour explants were labeled for 30 minutes with H^3TDR, and then were reincubated for varying periods of time (see Table I). One group labeled at 72 hours was washed in a change of nutrient medium for 5 minutes before being reincubated. In the other 72-hour group, the labeled assemblies were reincubated in fresh medium containing unlabeled thymidine at 1000 × by weight the labeling concentration. At each reincubation time, groups of 3 cultures were fixed and autoradiograms were prepared on 3-μ sections. Counts

of total mitotic figures and labeled mitotic figures were made on 12 to 24 sections taken from the thickest, central part of each culture.

BUDR was administered to intact explants by replacing the culture medium with fresh medium containing BUDR at 10 to 300 μg per ml (no concentration-dependent effects were noted). For cultures treated longer than 24 hours, fresh analogue-containing medium was added after each 24-hour period of incubation. In groups in which analogue treatment was discontinued, explants were washed for two 5-minute periods in fresh medium before being reincubated.

of cells is clearly distinguishable by 48 hours *in vitro* and persists at least until 5 days, particularly in the peripheral zone or cortex. The central zone may show proacinar arrangement, or some of the cells may be packed closely together in an apparently unorganized mass. The appearance of these relatively unorganized cells, their low thymidine incorporation as indicated by grain density (see below), and their later failure to form zymogen granules make it likely that they are primarily non-exocrine.

It is in acini near the center of the explant that

TABLE I

Numbers of Standard Explants Labeled and Fixed at Varying Times

Labeled at	Fixed at 0	24	48	72	74–85	96	120	144	168
hrs. of culture	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*	*hrs. of culture*
0	6								
24		16							
48			7			2	2		
72				16	24	16	13		
96						9	6		
120							29		
144								3	
168									3

Each explant (13-day embryonic salivary gland mesenchyme/quarter piece 11-day embryonic pancreatic epithelium) labeled for 1 hour with 0.4 ml nutrient medium containing H^3TDR, 10 μc/ml, at 38°C, in humidified, 5 per cent CO_2 atmosphere. At least 2 autoradiograms prepared for each explant (exposures 3 to 5 and 10 to 15 days).

RESULTS

Epithelial Maturation in vitro and in vivo

Maturational changes in the pancreatic epithelium during culture, for the experimental system under study, are described in the preceding paper (16). The following facts are important to emphasize for present purposes. The explant, which is one-quarter of the epithelium from one rudiment, consists of a mass of cells interpenetrated by branching luminal cavities, which in the whole rudiment were probably in communication with the central lumen. Increase of mass during 5 days in culture is the result of cell growth and division. The morphogenetic pattern concomitant with growth is not fully understood, but it is clear that most cells continue to be associated with a lumen as the exocrine acini form. Proacinar arrangement

zymogen granules (appearing dense under phase contrast microscopy) are first seen, usually in cultures fixed at 96 hours (13 of 17 cultures examined). The distribution of cells containing such granules is represented in Figs. 4 to 6, based on study, with phase-contrast optics, of autoradiograms and of routine sections close to those used for autoradiography. Beyond the 4th day, and up to at least the 7th day, both the number of granules in individual cells and the number of granule-containing cells increase. The increase tends to occur sequentially from the central region to the most peripheral cortical cells (*cf.* Figs. 4 to 7). At 5 days (Figs. 7, 8), for example, most cells on the inner side of the cortical acini are packed with zymogen granules, while in the outermost layers some cells contain smaller numbers of granules and others are devoid of identi-

fiable zymogen. The impression is of a centro-peripheral gradient of exocrine differentiation in the explant.

Comparison of the epithelium *in vitro* with that in the developing embryo suggests the main difference to be the more obvious three-dimensional branching of the epithelium into the surrounding mesenchyme *in vivo* (Fig. 9). In correspondence with the observations *in vitro*, in which zymogen first can be recognized optically on the 4th day, granules (appearing dense under phase-contrast microscopy) are seen in about half of the pancreatic lobes examined from 15-day embryos. In these instances, the granules usually are seen in cells of the deeper lying acini, acini which only rarely contain mitoses. In glands lacking zymogen, there are numerous mitotic figures in both the peripheral and deeper lying acini of the lobes. At 16 days, abundant zymogen granules are present in central acini near the base of the lobe. Granules are found in fewer cells and in smaller numbers per cell in the more peripheral acini. Mitoses are still frequent in the most peripheral acini, even when many nearby cells contain some zymogen granules. In rare instances, zymogen is clearly visible in dividing cells. Between 16 and 18 days large quantities of zymogen accumulate in all of the exocrine cells (Fig. 11), and mitosis no longer is seen.

Thymidine Incorporation in vitro

Figs. 1 to 5 show typical cross-sections through the thickest portion of cultures fixed after 1 to 5 days *in vitro*. After 1 day, labeled nuclei occur nearly everywhere in the explant, although there is some tendency toward greater frequency and intensity of labeling peripherally. It is to be noted that the presence of mesenchyme clearly affects the labeling pattern at 1 day. This was determined by 2 experiments, 1 of which is reported here (Table II). In each experiment an analysis was performed on the central 10 to 12 sections of explants cultured in the presence and absence of mesenchyme (8 each). The average cross-sectional area of such sections in the absence of mesenchyme was estimated at 3790 μ^2, in contrast to 10,090 μ^2 in the presence of mesenchyme. The number of labeled nuclei per unit area (1000 μ^2) in the presence of mesenchyme was nearly twice that in its absence. Grain densities over the labeled nuclei were approximately equal in both instances. Although these data are only semi-quantitative, they indicate strongly that thymidine incorporation and growth in pancreatic epithelium during the first 24 hours are stimulated by the presence of mesenchyme.

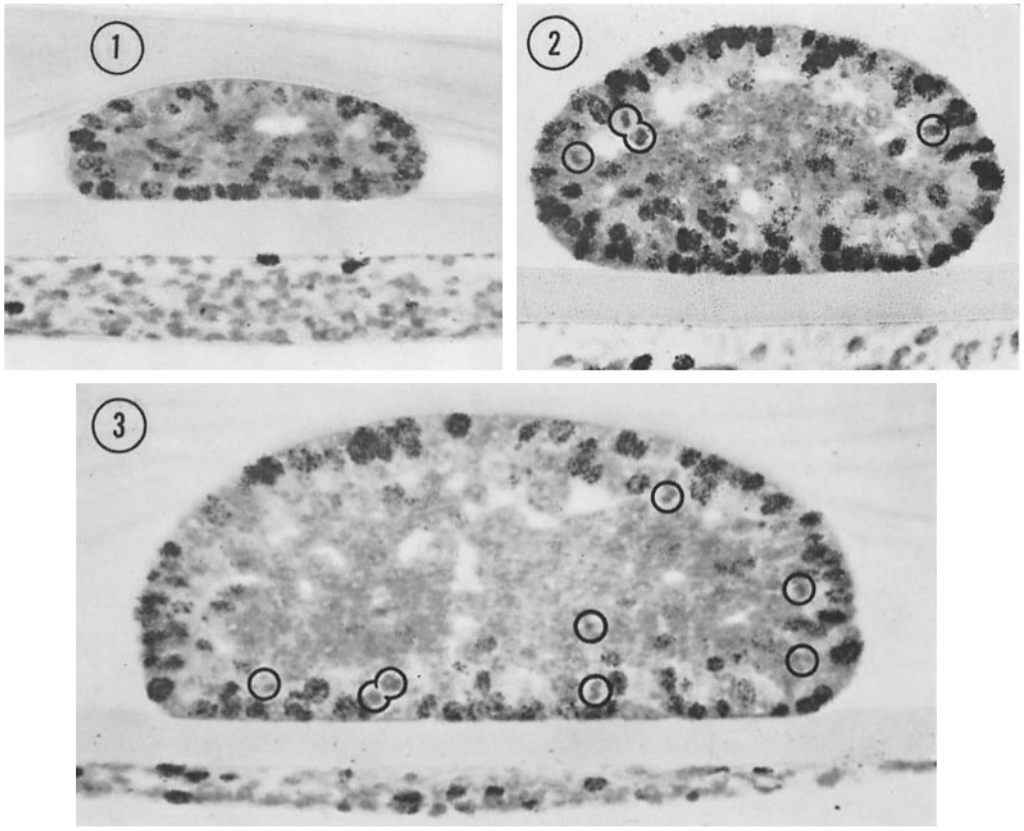
The tendency to peripheral localization of labeling suggested in 24-hour cultures is considerably more marked 1 day later, and is sharply defined by the 3rd day (Figs. 2, 3). On the 3rd day, and increasingly at later stages (Figs. 4, 5), labeling of central nuclei is rare and the density of grains over nuclei is noticeably less in cells below the surface than at the surface. At 4 days and beyond, the impression is of labeling confined largely to nuclei in the surface layer and the region immediately below. At 5 and 6 days, as zymogen granules become increasingly prominent in the more peripheral acini, the frequency of labeled nuclei even in the peripheral cells decreases. Over-all, the observations suggest a centro-peripheral sequence of restriction of thymidine incorporation, moving just ahead, in time, of the similar sequence in first appearance of zymogen referred to above.

It is obvious that interpretation of these thymidine-incorporation data is complicated by the possibility that they reflect differential availability of H^3TDR to central and peripheral cells. The following observations are relevant in evaluating this possibility. Four 5-day cultures labeled for 4 hours instead of 1 hour showed typical peripheral labeling, with no increase in the number of labeled internal nuclei despite the very great increase in density of grains over the peripheral nuclei. Other cultures (3 each at 3, 4, and 5 days) were cut across their center, perpendicular to the filter, in order to expose the internal differentiating population directly to the labeling medium. Such explants failed to show any significant difference in their labeling pattern as compared with typical controls. Similarly, 11-day epithelia implanted into 5-day cultures showed characteristic heavy labeling, in one favorable instance where the heavily labeling fresh epithelial face was immediately adjacent to sparsely labeling internal cells of the culture. Finally, whole 11-day epithelia, clotted on a filter and labeled immediately, show, around the central lumen, a region free of labeled nuclei. When an epithelial rudiment was quartered, and the quarters clotted individually on filters prior to labeling, 3 of the 4 quarters showed a small, non-labeling zone *at their periphery*—presumably

representing a portion of the central non-labeling population of the intact rudiment. These observations, while not fully conclusive, suggest that the peripheral labeling is not a gross artifact resulting from failure of penetration of the tritiated thymidine into the culture mass. The best assurance, however, that there is actually a peripheral population of cells undergoing rapid division comes from observations reported below on the subsequent history of labeled nuclei.

Thymidine Incorporation in Freshly Isolated Pancreas

Freshly isolated pancreatic rudiments labeled *in vitro* show patterns of incorporation similar to those of the corresponding culture stages. Ten- to 12-day rudiments have labeled nuclei throughout their mass, though, as noted above, fewer such nuclei seem to occur near the lumina in which mitosis takes place. Labeled nuclei are frequent in the mesenchyme around the epithelium, and



FIGURES 1 to 5 Hematoxylin-stained autoradiograms (H^3TDR). Cells in mitosis represented by circles. Regions with easily identifiable zymogen granules indicated by broken lines. *ca.* × 280.

FIGURE 1 24-hour explant. Labeled nuclei occur throughout the epithelium, but in somewhat greater frequency peripherally. In this section no mitoses were seen; nearby sections contained up to 3 mitotic figures.

FIGURE 2 48-hour explant. A distinct peripheral incorporating layer is evident, as well as internal cells covered by fewer grains. Luminal cavities are becoming prominent.

FIGURE 3 72-hour explant. The peripheral incorporation pattern is more marked, and, in addition, a central, grain-free region is found in most explants.

in the covering visceral peritoneal layer. By 13 days, and increasingly at later stages, the frequency of heavily labeled nuclei is greatest in the outer acini. Fewer labeled cells, most with lower grain density, occur in the internal acini. In these respects, the pattern resembles that of a 3-day culture. The cells of ducts and islet tissue are infrequently and lightly labeled in all stages examined (11 to 18 days).

Labeled nuclei continue to be preponderant at

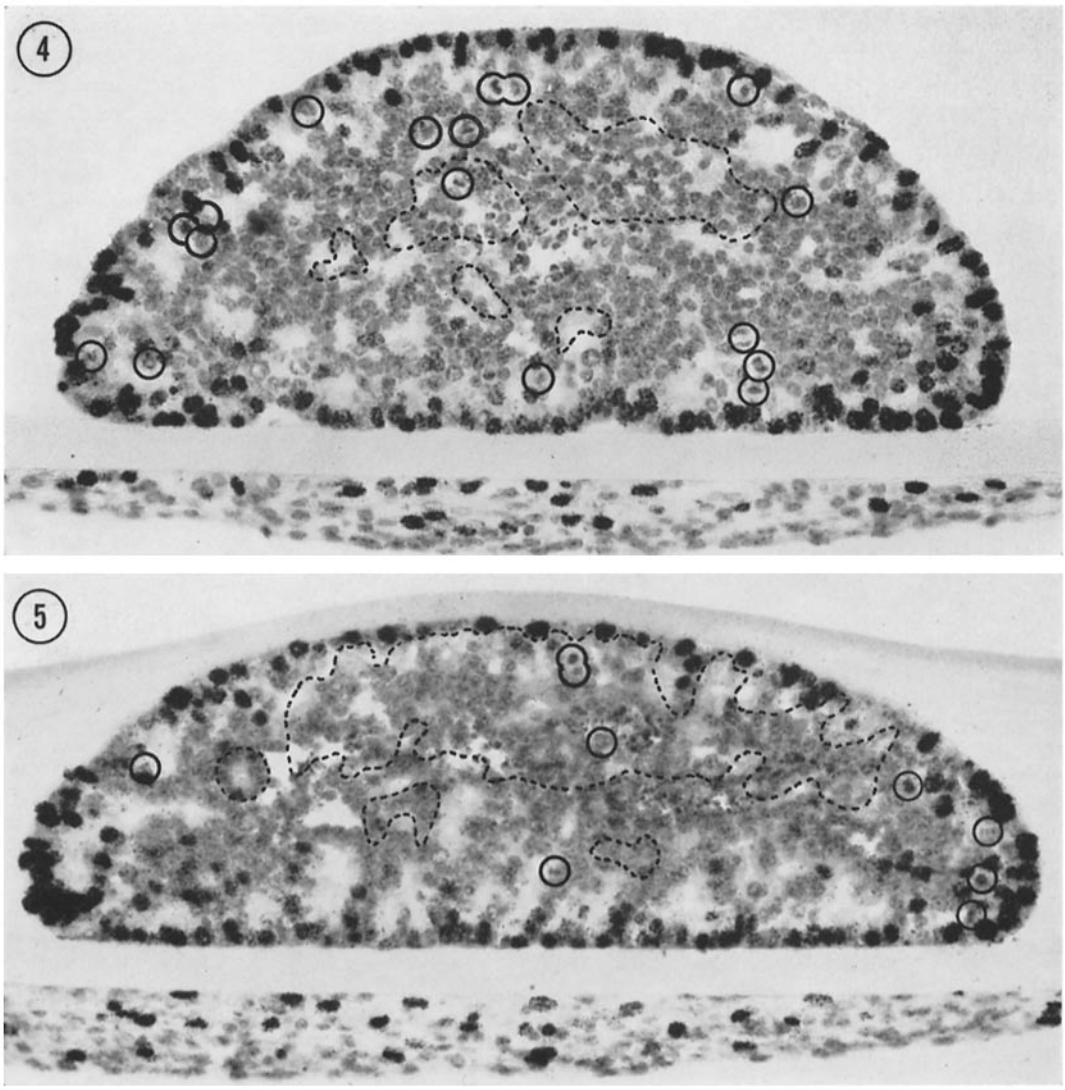


FIGURE 4 96-hour explant. A marked increase in mass has occurred. Only limited numbers of internal cells incorporate thymidine, and those at a rate yielding few developed grains per nucleus. Zymogen granules are found in the central areas (outlined with broken lines). No granules are seen in more peripheral acini, where H^3TDR incorporation is still heavy.

FIGURE 5 120-hour explant. The number of labeling peripheral nuclei per unit length of the outer surface has decreased in most sections. In contrast to the situation in Fig. 4, zymogen granules are found in peripheral acini and cells. Whereas the heaviest distribution of zymogen in this section is nearer the clot-surface of the epithelium, other sections or explants may show their heaviest accumulation elsewhere in the epithelium.

the periphery through the 16-day stage, at which time small amounts of zymogen are found in some cells of the outer, labeling acini (Fig. 10). Small segments of carefully dissected pancreas, including both peripheral and central acini, continue to show the differential incorporation despite direct exposure of the central acini to the thymidine solution. Between 16 and 18 days of gestation, H^3TDR incorporation virtually ceases, not only in the exocrine cells which have accumulated zymogen, but also in the cells of the stroma and ducts (Fig. 11). It seems clear that the normal pancreas developing *in vivo* shows a centro-peripheral sequence of thymidine incorporation and of zymogen appearance similar to that occurring during development *in vitro*.

TABLE II

Numbers of Labeled Pancreatic Epithelial Nuclei after 24 Hours in Vitro

	Number of labeled nuclei per 1000 μ^2 in	
	Epithelium alone	Epithelium with mesenchyme
	2.24	5.04
	2.38	5.03
	1.58	5.01
	2.34	4.78
	3.60	4.36
	3.22	5.51
	2.24	4.26
	3.19	4.95
Means	2.59	4.68

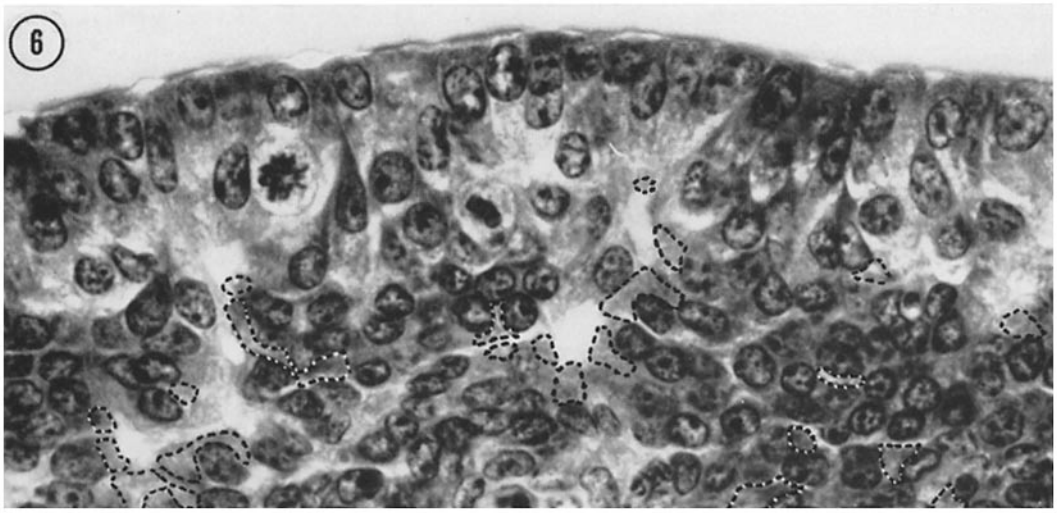
Each figure is the mean from counts of 10 to 12 mid-sections from a single culture. The figures are estimates only, because of the following reasons: 1) the rounded corners of explants were ignored so that the adjacent tissue-free corner of the counting grid was included in the measured area; 2) for figures in column 2, underestimate of the epithelium counts is probable because of non-labeling areas in the center of the tissues, and because so many closely associated nuclei were labeled that low grain-count nuclei were certainly overlooked. Difference between means highly significant; $P < 0.001$. $t = 65$.

Mitotic Behavior in Relation to Thymidine Incorporation and Zymogen Synthesis

The distribution of mitotic figures in cultured epithelium is indicated in Figs. 1 to 5. In freshly prepared epithelia or 24-hour cultures, mitoses are scattered throughout the interior of the epithelium, and, in most cases, are in cells adjacent to lumina. By the 2nd or 3rd day in culture (Fig. 3), it is clear that most mitoses occur in the zone immediately below the heavily labeled periphery. Occasionally, dividing cells are seen adjacent to the filter, and quite rarely they occur centrally. In these cultures fixed immediately after labeling, none of the mitotic figures are labeled.

A series of cultures was prepared in which labeling at 72 hours was followed by reincubation for varying periods prior to fixation. Controls, fixed without reincubation, contained most of the H^3TDR-marked nuclei in the outer layer (Fig. 3). After 2 hours of reincubation, the over-all distribution of labeled nuclei was not markedly altered, but grains were present over some of the mitotic figures located in the subcortical, mitotic zone. With reincubation for 4 and 7 hours, the pattern of labeling changed significantly (Fig. 12). Many labeled nuclei now were present below the surface. Some were large and round with a clear, non-staining (hematoxylin) center and with silver grains tending to be concentrated over their periphery. In addition, particularly at 7 hours, smaller nuclei were marked by intense hematoxylin staining and small dense masses of silver grains over them. These presumed daughter nuclei of earlier labeled cells sometimes occurred in the periphery, and sometimes deeper within the explant. Their frequency decreased with further reincubation, just as lightly labeled nuclei became more abundant in the same regions.

To obtain data on the mitotic cycle, counts were made of total mitotic figures and of labeled mitotic figures in 10 to 24 sections taken from the thickest, central part of cultures of increasing reincubation times. Total mitoses counted ranged from 24 to 102 for a single culture. The data on percentage mitoses labeled are given in Fig. 13. They indicate the following: (*a*) no thymidine is incorporated by cells undergoing mitosis at the time of labeling; (*b*) by 2 hours of reincubation, over 50 per cent of the mitoses are labeled, suggesting that the G2, or post-DNA-synthetic period, is quite short; (*c*) between 4 and 8 hours of reincubation, virtually 100 per cent of the mitotic figures are labeled, while by 12 hours, the percentage has dropped again considerably (when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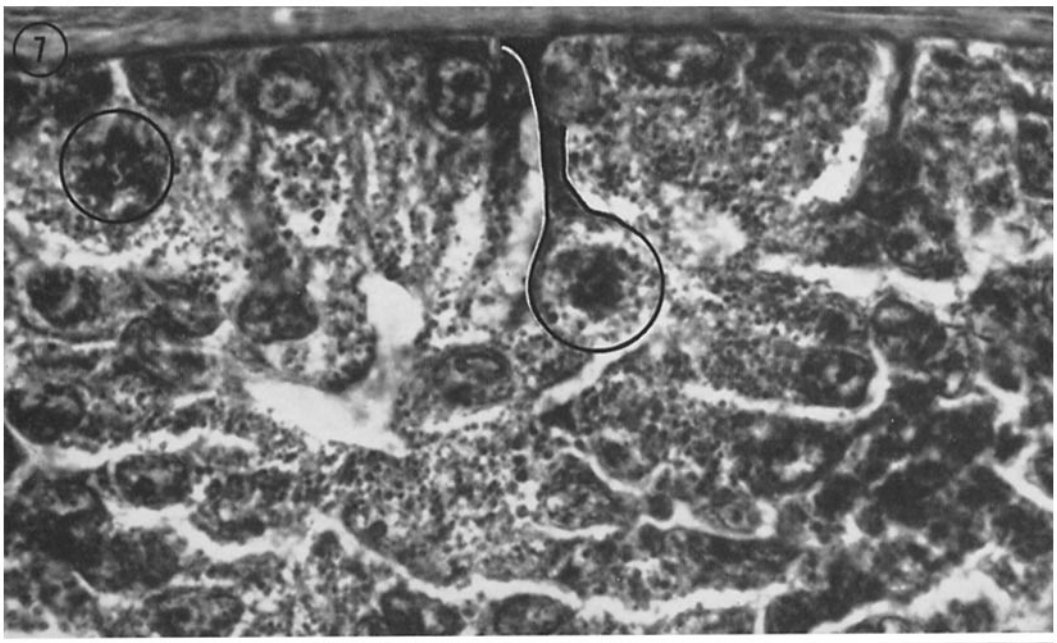
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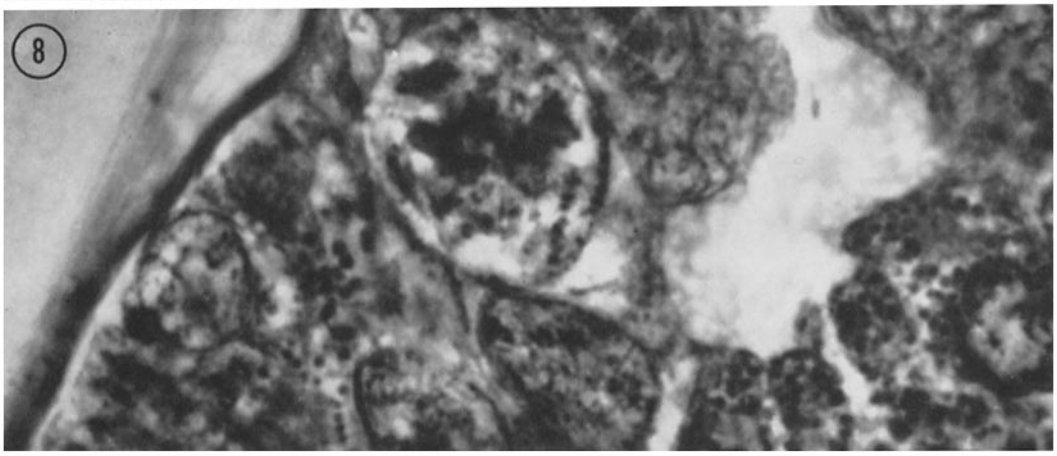
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precautions were taken against incorporation of residual label). The curve of labeled mitoses suggests that the synthetic (S) period for DNA is approximately 10 hours.

It is noteworthy that it is not uncommon to find, in 5-day cultures and beyond, metaphase plates in cells located in peripheral, zymogen-containing acini. In some explants, division figures were seen in cells containing relatively sparse clusters of eosinophilic granules (which appear dense under phase-contrast microscopy) (Fig. 8). Such figures never occur in zymogen-packed cells internal to the cortical layer.

The observations on the relation of mitosis to thymidine incorporation and to zymogen-granule production may be summarized as follows: During the first 48 hours in culture, thymidine-incorporation, and presumably DNA synthesis, becomes concentrated in nuclei of the peripheral layer of cells. Actual division of these nuclei occurs in a zone removed from the epithelial surface, usually in cells associated with a luminal cavity. Whether the shift is due to nuclear migration within cells, or actual movement of whole cells is not certain. There are indications that at least some of the deeper lying cells retain connection with the surface *via* long, fiber-like processes (Fig. 6). In any event, the products of division appear either to return to the peripheral layer for renewed DNA synthesis, or to remain centrally where DNA synthesis is at least much less likely. The accumulating central population of cells is the first to undergo zymogen synthesis, and the process spreads peripherally. Some cells which have begun zymogen synthesis can undergo division, but it is not clear when the DNA of such cells was replicated in relation to the initiation of granule synthesis. Cells which are firmly committed to zymogen synthesis show no thymidine incorporation or mitotic activity.

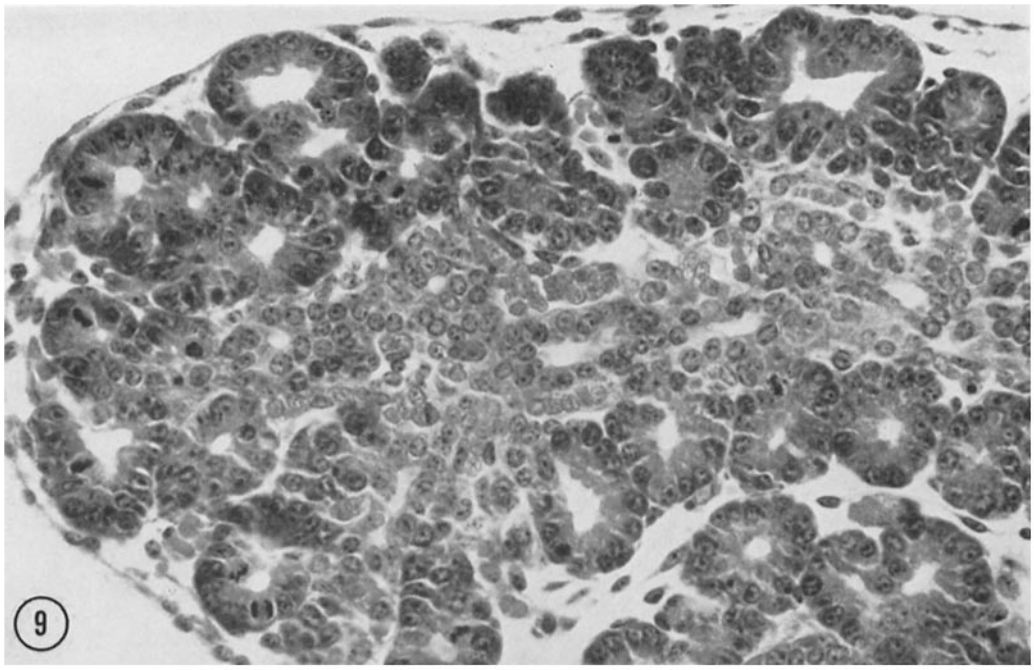
The Central, Non-Labeling Population

The accumulation and subsequent behavior of the central non-labeling population of cells which undergoes differentiation first was studied further in a series of cultures which were labeled at 2, 3, and 4 days and reincubated for varying periods. Sixteen compact cultures, labeled on day 3 and fixed on days 4 or 5, showed a central region of grain-free cells (Fig. 14) in every instance. The size and shape of the region varied; in some instances, it appeared to be completely acinar, in others, mixed acinar and duct (as defined above). The non-labeling island was completely surrounded by abundant labeled cells. Among these labeled cells of some sections, there appeared to be a decreasing centro-peripheral gradient of labeling; *i.e.*, more centrally located nuclei tended to be covered by higher grain-densities than peripheral ones. The over-all distribution in these explants would occur if the centermost unlabeled cells had ceased thymidine incorporation prior to labeling, if the somewhat more peripheral, heavily labeled cells

FIGURE 6 Hematoxylin-eosin section of a 96-hour–labeled culture. Zymogen granules (cells outlined with broken lines) are found only rarely in the outer cells. Quantities of granules per cell are greatest in the internal (lower) exocrine cells. Note the mitotic figures and the cell, just to the right of a prominent metaphase plate, which apparently retains a connection with the epithelial surface *via* a cytoplasmic extension. × 870.

FIGURE 7 Phase-contrast, hematoxylin-eosin section of 120-hour–labeled culture. Zymogen granules are seen scattered throughout the peripheral acinar cells. Although unclear, two mitotic figures are present, one in a cell which appears to be connected with the epithelial surface when examined microscopically. Such inspection also indicates that the quantity of zymogen granules is much greater in the lower, more internal exocrine cells. × 1600.

FIGURE 8 Phase-contrast, hematoxylin-eosin section of 120-hour–labeled explant. The mitotic cell contains eosinophilic granules (appearing dense under phase contrast microscopy) near its apical (luminal) surface. Other optical sections through this cell reveal more granules than are here evident. Note the adjacent outer epithelial cells with a small number of zymogen granules, and, across the acinar lumen, cells with larger numbers of granules (see text). × 2750.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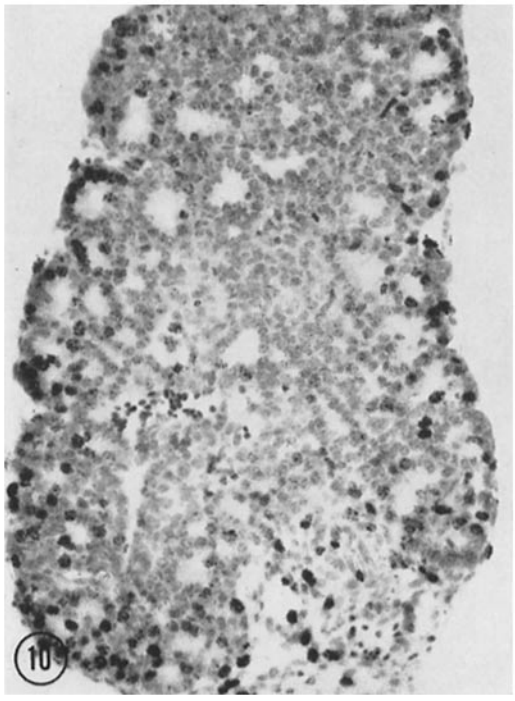
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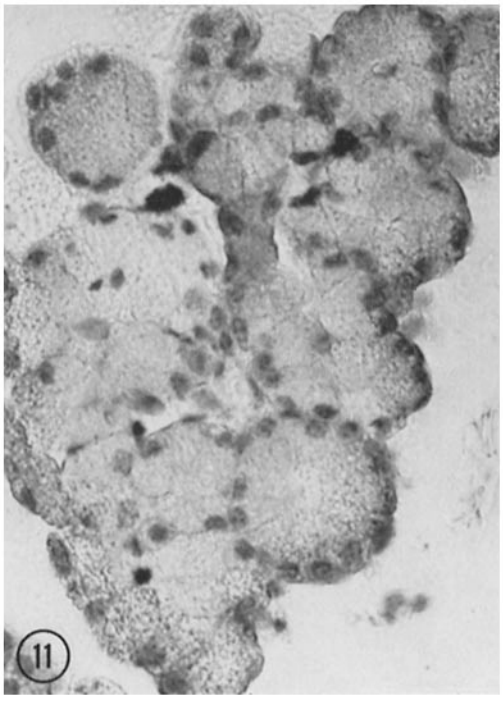
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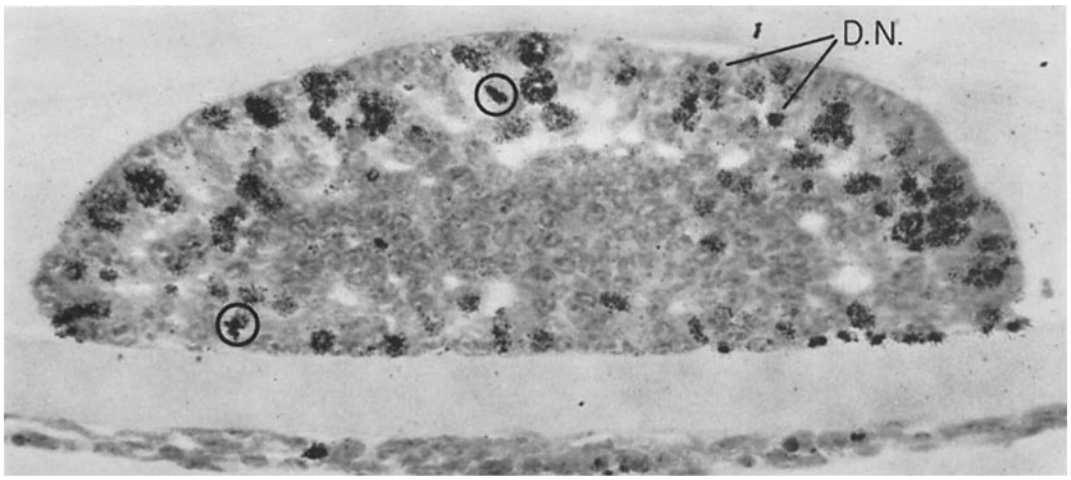

FIGURE 12 Hematoxylin autoradiogram of a culture labeled at 72 hours, washed, and then reincubated for 7 hours in the presence of carrier thymidine. In contrast to the situation in controls fixed immediately after labeling, many radioactive nuclei appear below the cortical layer of the epithelium. Other sections of this and other explants show greater areas of unlabeled nuclei in the outermost cell layer. At this time (7 hours), 100 per cent of the mitotic figures are labeled. The 2 large, labeled nuclei at the top-center are thought to be in a stage of prophase. Small, dense accumulations of grains in various places (DN) may be daughter nuclei. × 430.

are products of the first division after labeling, and if the more moderately labeled and more peripheral cells arose after 2 or more divisions, etc. That the turnover rate in the peripheral proliferative population is high is suggested by the very large number of labeled cells present below the periphery after only 24 hours of reincubation. Scattered among these abundant, lightly labeled nuclei is a small percentage of nuclei covered by much higher grain-densities (equivalent to those found in cultures fixed immediately after labeling). Such apparent in-

FIGURE 9 Hematoxylin-eosin section of pancreas of 16-day embryo. The central duct system runs from right to left. Examination of the acini with higher magnification reveals many zymogen granules in most of the acini from the middle of the photograph to the right. Few mitotic figures are seen in these acini. In contrast, the acini to the left of center contain cells with few or no zymogen granules. Mitosis is quite common in this region, and is seen in some cells with small numbers of zymogen granules. × 425.

FIGURE 10 Hematoxylin autoradiogram of pancreas of 16-day embryo. The peripheral acinar clusters show frequent and heavy thymidine incorporation. This presumably relates to the heavy mitotic activity seen in Fig. 9. Internally, there is little incorporation in acinar cells which contain more zymogen granules, or in duct or islet tissue (when present). Smaller pieces of 16-day gland cut with the internal region exposed directly to the labeling solution continue to show an absence of thymidine-binding (as for the older stage in Fig. 11). × 205.

FIGURE 11 Hematoxylin autoradiogram of normal pancreas of 18-day embryo. Only 7 labeled nuclei are seen in this typical group of zymogen-packed acini. In most such cases, at 18 days, closer inspection shows that the labeled nuclei are in mesenchymal stromal cells that lie between the acini. Mitotic figures have not been found in exocrine cells at this late stage of gestation. × 460.

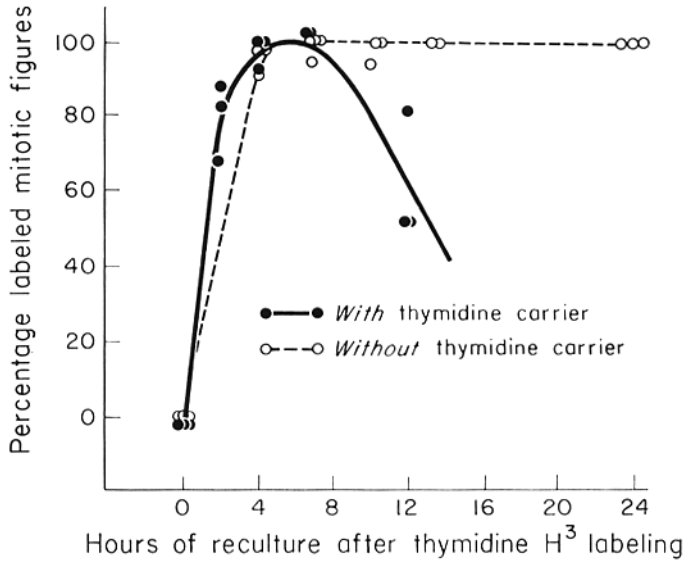

FIGURE 13 Percentage of labeled mitotic figures in explants labeled at 72 hours (0 time) and reincubated for varying periods. Filled circles indicate cultures reincubated with carrier thymidine; open circles indicate cultures reincubated in normal medium after washing. Each point represents the average count of at least 12 sections in the central part of an individual epithelium (thus, the 94 per cent point at 7 hours is for 33 labeled figures in 35 counted). The failure of explants not "chased" with cold thymidine to fall below the 100 per cent level is attributed to continued low-level incorporation during the reincubation period (see text).

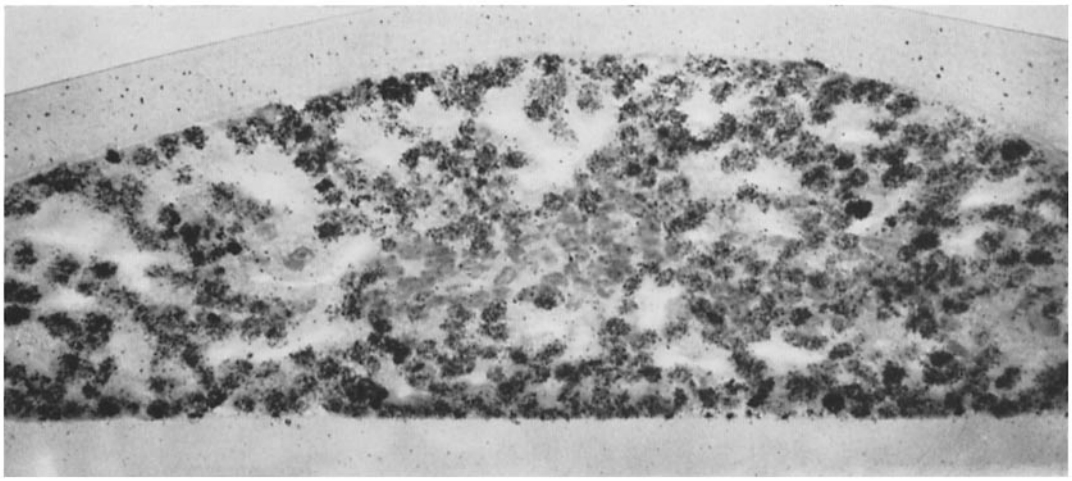

FIGURE 14 Hematoxylin autoradiogram of a culture labeled at 72 hours, washed, and then reincubated until fixed at 120 hours. Zymogen granule distribution is normal for a 5-day explant, and many of the labeled cells are packed with granules. Note the small region, near the center of the explant, that apparently failed to incorporate the DNA-precursor during the labeling period. The region has remained free of labeled cells during the 48 hours of reincubation. Surrounding this region are the many descendant cells from the original peripheral labeled population (as in Figs. 3, 12). × 400.

frequently dividing cells occur randomly throughout the peripheral and subjacent acini, and frequently in proximity to the central label-free zone.

The non-labeling central population appears to reach significant size between the 2nd and 3rd day, and to continue its increase at least until the 4th day. When four 2-day cultures were labeled and reincubated until the 5th day, lightly labeled (presumably because division occurred since labeling) and sometimes contain small numbers of zymogen granules. The situation contrasts with that in cultures labeled at 3 days and fixed at 5 days in which the inner cells of the peripheral acini are both zymogen-packed and labeled. The facts clearly fit the assumption that the central unlabeled population increases in size by addition of cells at its periphery, and

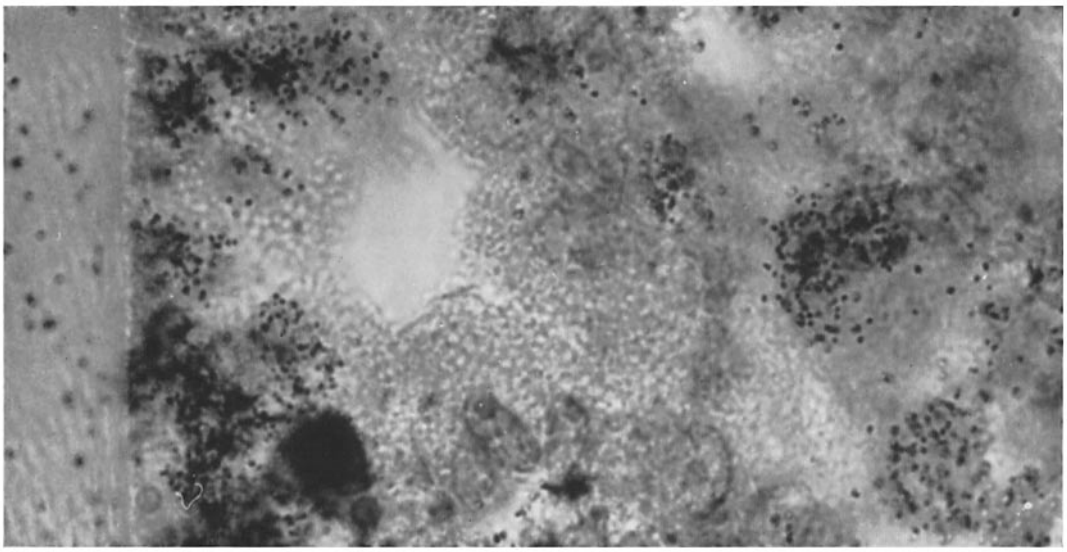

FIGURE 15 Hematoxylin autoradiogram of a surface acinus from an explant labeled at 96 hours, washed, and reincubated until 120 hours. Cells on the proximal (right) side of the acinus are unlabeled and their cytoplasm is packed with zymogen granules (light, refractile spots). The outer portion of the acinus is composed of daughter cells of the population labeled at 96 hours. Zymogen granule formation has occurred in some of these cells, but fewer granules are present than in the unlabeled "older" cells across the lumen. × 1680.

a recognizable label-free zone was present in only one of the four cultures. Conversely, when 4-day cultures were labeled and reincubated until the 5th day, a label-free center was present in all 6 cultures examined. These centers were larger in absolute size, and also larger relative to the epithelium as a whole, than the equivalent centers in cultures labeled and reincubated at 3 days. In many instances, in the cultures labeled at 4 days (Fig. 15) the *peripheral* acini show an interesting difference between their inner and outer sides. Cells on the inner side are generally unlabeled but packed with zymogen granules (Fig. 14), whereas cells on the outer side are that cells which later join the non-labeling population differentiate later, *i.e.*, in a centro-peripheral gradient.

Effect of 5-Bromodeoxyuridine

Bromodeoxyuridine (10 to 300 μg/ml) was incorporated in the culture medium of groups of cultures during the following periods: 0 to 24, 0 to 48, 24 to 48, 48 to 72, and 72 to 120 or 72 to 144 hours. At times other than those of BUDR treatment, the cultures were on standard medium and all were maintained until at least the 5th day. Of 21 cultures treated for any part of the period 0 to 72 hours, none showed zymogen

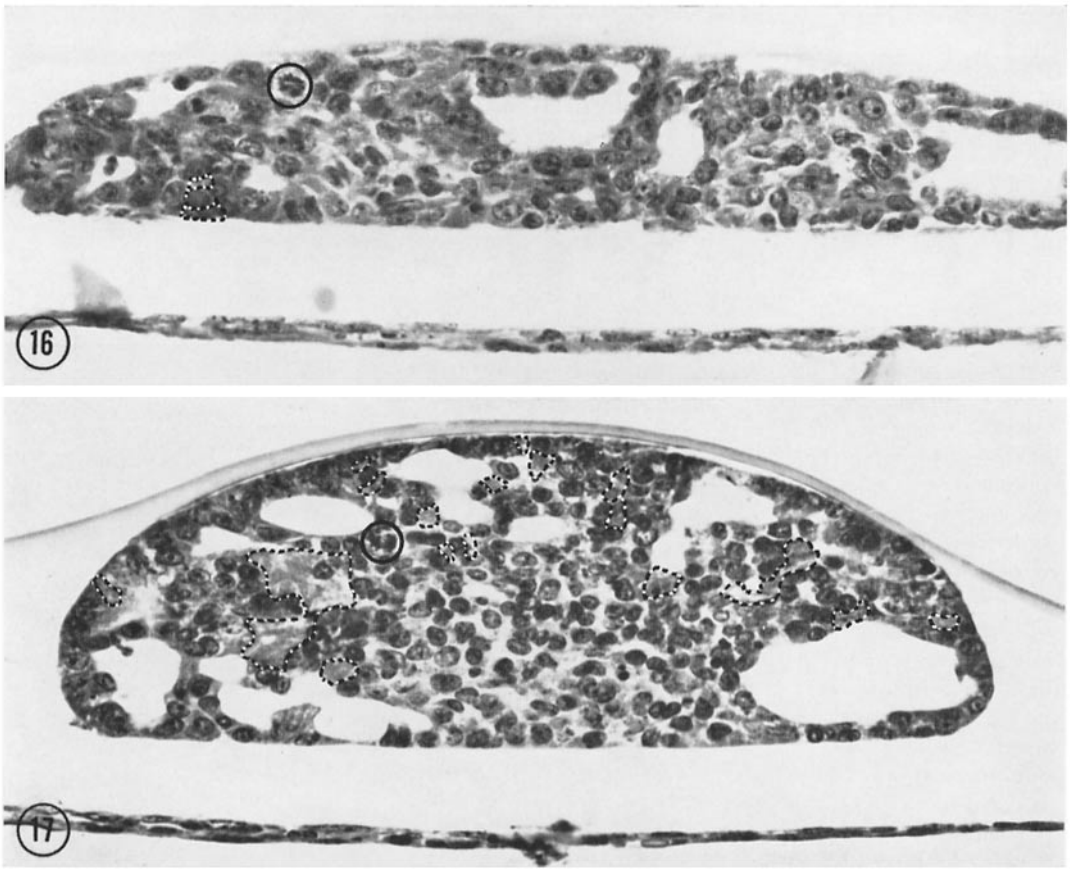

FIGURE 16 Hematoxylin-eosin section of a culture treated with BUDR from 48 to 72 hours, washed, and reincubated until 120 hours. In this explant, approximately 12 zymogen-packed cells were discovered, one of which is indicated in this section. Most cells appear to be quite healthy but undifferentiated with regard to granule formation. Note the mitotic figure (in circle), here appearing in a cell 48 hours after BUDR treatment was completed. × 415.

FIGURE 17 Hematoxylin-eosin section of an explant treated with BUDR from 72 to 120 hours. Zymogen-containing cells, indicated by broken-line outlines are remarkable in that almost all of them are packed with zymogen granules. They seem to be at a more uniform, fully differentiated state than do many acinar cells in control explants. The remainder of this explant seems to be healthy but undifferentiated, and resembles the bulk of tissue cells found in cultures treated at earlier times (as Fig. 16). × 430.

formation identifiable in the living state at 5 or 6 days. Abundant zymogen was visible in all 14 untreated control cultures carried simultaneously. The BUDR-treated cultures were healthy and translucent during the culture period, though they remained smaller than controls. Growth inhibition was particularly marked in 6 cultures treated during the first 2 days, and was hardly apparent in those treated only from 48 to 72 hours (Fig. 16). Sections of the BUDR-treated epithelia, stained with hematoxylin and eosin or with Feulgen reagents, confirmed the absence of zymogen granules in 16 of the 18 cultures examined. In two cases, both in the 48- to 72-hour group, scattered individual zymogen-containing cells were observed, 4 in one culture and 12 in the other. No mitotic figures were observed in cultures treated during the first 2 days, whereas those cultures treated only on the 2nd or 3rd day showed a few mitotic figures. No obvious chromosome breaks or abnormalities were observed in these mitotic cells. The reduced mitotic activity ap-

pears to be correlated with the small size of the explants. The cytoplasm of the BUDR-treated cells showed little or no eosinophilia, such as is usually associated with the predifferentiative stages of pancreatic exocrine cells, both *in vivo* and *in vitro*. Over-all, the observations suggest that exocrine differentiation can be prevented by BUDR treatment for any 24-hour period of the first 3 days in culture, and that toward the end of the 3rd day the first BUDR-insensitive cells are appearing.

The behavior changes markedly with treatment beyond the 3rd day. In cultures treated continuously from 72 hours on, the central region at 5 days was dark or opaque in transmitted light, the usual sign of considerable zymogen formation in control cultures. Around the central dark region there was an unusually wide translucent border or cortex, frequently broken up into vesicles or atypical acini. In 3 cultures carried to the 6th day, neither the dark center nor the wide, clear border increased noticeably in size. Sections of cultures treated with BUDR during these later times showed a number of atypical vesicles and acini with inflated lumina (Fig. 17). Despite this, acinar masses of cells occurred which were packed with eosinophilic granules which appeared dense under phase-contrast microscopy. The acini were scattered among larger numbers of granule-free cells, which were particularly concentrated toward the periphery of the section, presumably corresponding to the translucent border seen in the living state. Of particular interest were solitary zymogen-containing cells occurring here and there in the otherwise undifferentiated areas.

DISCUSSION

The general conception stemming from these data on the behavior of pancreatic epithelium, under the experimental conditions described, is as follows. Soon after explantation the population of cells making up the fragment develops a peripheral proliferative "compartment," or subpopulation, in the same developmental state, whose cells are synthesizing DNA. Their nuclei at this stage are at the surface of the explant, but the cells undergo division while their nuclei are removed from the surface. By virtue of its very high rate of replication, the proliferative compartment both expands outward—increasing its own cell number, area, and volume—and, concomitantly, leaves behind a central subpopulation within which there is little division but rapid differentiation. The differentiative compartment increases in cell number by peripheral recruitment from the proliferative compartment; hence there is a centro-peripheral wave of differentiation. Once started, differentiation invades and reduces the size of the proliferative compartment. The system would be expected to be mature, *i.e.*, showing characteristics of the final differentiated state and showing no net growth, when all of the most peripheral cells have undergone differentiation.

Clearly, the validity of this conception rests heavily on the observation of concentrated peripheral labeling when cultures are fixed immediately after exposure to tritiated thymidine. It is important, therefore, to consider the possibility that centrally located cells—for some undefined reason—are failing to incorporate the label even though they are, in fact, synthesizing DNA and undergoing division. The following considerations appear to justify the conclusion that this is not the case. (*a*) Increased labeling time affects the intensity of labeling but not the distribution of labeled nuclei; (*b*) explants which have been cut so as to expose their low labeling centers directly to the labeling solution show unchanged patterns of labeling; (*c*) mitotic figures are rarely seen in the central population (though this could be due to movement peripherally for division); (*d*) labeled mitotic figures increase to 100 per cent and are maintained at that level for 4 to 7 hours after labeling (this argues strongly that no significant number of cells can be preparing for division without incorporating thymidine label); and (*e*) tritiated uridine and tritiated leucine are incorporated in the nuclei and cytoplasm, respectively, of the central cells after they will no longer incorporate thymidine, indicating that there is no general barrier to penetration of precursors (unpublished data). These facts, together with the generally similar behavior of freshly isolated pancreatic rudiments, and the consistency of the observations on granule synthesis and the BUDR effect (see below), lead to the provisional assumption that the peripheral pattern of thymidine labeling *in vitro* accurately reflects cell activity.

The presence of lightly labeled nuclei in inner acini where mitoses are not seen is puzzling. If one accepts for the moment the proposition that

excess H^3TDR is available to these cells, two main interpretations of the low grain counts seem plausible. First, the rate of DNA synthesis may be reduced (length of S phase increased) in the predifferentiative population. Data which can be interpreted in this way have been presented for the maturation of stem cells to type B spermatogonia (22), for maturation of bone marrow elements (20), for maturing blood cells of the erythroid series (3), and for developing plasma cells (21, 23). If this is, in fact, the case in the pancreatic epithelium, the absence of actual mitoses in these acinar regions still must be explained. As a second alternative, Pelc (25) has hypothesized that a small percentage of differentiated cells may incorporate tritiated thymidine at a low rate even though they are not preparing for mitosis. Thus, during postmitotic differentiation in the pancreas small quantities of thymidine (which become fixable) might be required in the nucleus for some unknown process related to specialized synthesis.

The notion that nuclei which synthesize their DNA while in one position subsequently undergo division in another position (see p. 431) is in accord with analyses of other proliferating systems, including the neural tube and retina of the mouse (30, 31) and the chick (10), and the skin of the chick (Wessells, unpublished data). In the latter instance, incorporation of thymidine is limited to the basal layer, but mitotic figures are observed both in the basal layer and immediately above. Subsequently, nuclei with reduced grain count, presumably resulting from division, are found both more peripherally and in the basal zone.

The precise relationship between the time at which DNA-synthetic cycles cease and overt zymogen accumulation commences in a given epithelial cell has not been defined by the studies reported on the pancreas. Zymogen granules are seen in most cultures at 96 hours, but only in cells interior to the peripheral DNA-synthetic and mitotic areas. In many of the cultures fixed at 120 hours the inner cells contain large numbers of granules but the peripheral cells are only lightly granular. Concomitant with this differentiative pattern there is a decrease in numbers of peripheral nuclei which incorporate thymidine. In a very few of the residual mitotic cells, zymogen granules are seen in the cytoplasm—a situation similar to that observed in the 16-day embryonic pancreas. In neither system are mitoses ever found in cells that have accumulated large numbers of zymogen granules. It is to be noted that the presence of a mitotic spindle in a cell containing some zymogen granules says nothing about the time at which preparation for division, including DNA-synthesis, occurred. It is precisely this which one would like to know, if one wishes to answer the question whether DNA synthesis and specialized synthesis can go on simultaneously in the same cell, *i.e.*, whether replication and translation of the genome are exclusive processes. The fact that mitotic figures are occasionally found in cells in the early stages of specific synthesis may be less significant, in this connection, than the fact that no mitotic figures are seen in the more fully differentiated cells filled with zymogen. More critically, however, one would like to examine the thymidine-incorporating behavior of cells in the earliest stages of specialized synthesis—which should be possible by the combined techniques of autoradiography and electron microscopy.

The results so far available on the pancreas agree roughly with those obtained by Stockdale and Holtzer on developing chick muscle cells (32, 33), and by Makela and Nossal on maturing plasma cells (21). The former authors never found thymidine incorporation in cells which reacted with antibody to myosin, and concluded that developing muscle cells do not simultaneously synthesize DNA and actomyosin. Makela and Nossal (21) demonstrate that thymidine incorporation, and presumably mitosis, halt before the time of maximum antibody production by antigen-stimulated rat plasma cells. They suggest, however, that a very small percentage of the early plasma cell population can synthesize DNA after the specific synthesis of antibody has commenced. Unfortunately, these studies fail to distinguish between *de novo* synthesis of antibody and release of pre-formed antibody. The latter possibility is suggested by Schooley's (29) finding that leucine-H3 is bound in greatest amount by the younger stages of plasma cells, and in lesser quantity by mature plasmacytes. In another analogous system, Young (38) has demonstrated an inverse relation between proliferative activity and cell specialization into osteoblast, osteocyte, and osteoclast. All of these cases serve to define more sharply the problem of division *versus* differentiation in embryonic cells. Specifically, it would seem that cell division can occur during the covert and early overt stages of

these differentiations, but that no mitosis is normally found in cells which show final characteristics of the differentiated state (*i.e.*, zymogen accumulation for exocrine pancreas). The data say nothing about the *capacity* for division of individual cells under altered circumstances.

In its temporal aspect the phenomenon of induction is related to the problem of the gradual stabilization of cells in a non-dividing, differentiated condition. As reported in preliminary form (13), zymogen synthesis in cultured pancreatic epithelium occurs only if the epithelium is in the presence of salivary mesenchyme for a minimum of 30 to 36 hours. Zymogen material will appear in some cells when the mesenchyme is removed after that time. The thymidine labeling and reculture experiments indicate that the first non-incorporating population is set up late in the 48 to 72-hour period of culture. Similarly, results from BUDR treatment suggest that the first BUDR-insensitive cells appear between 48 and 72 hours. Thus, in explants treated from 48 to 72 hours with the thymidine analogue, either no cells or only a very few cells per explant differentiate normally. A much larger population forms zymogen if treatment is delayed until the 72 to 120-hour period.

The interpretation of the BUDR results rests on a series of hypotheses. Evidence from microbial systems (39, 40), from various culture cell lines (4, 7, 14, 18), and from certain developmental systems (8, 17, 33), indicates that BUDR is incorporated into DNA during replication cycles. The analogue can replace up to 50 per cent of the DNA-thymine after one division of a HeLa cell population (14). Furthermore, after one division the BUDR is thought to reside in one strand of the DNA of the cell (7). The abnormality of the DNA produced by this incorporation process does not noticeably interfere with the early development of sand-dollar embryos (17), but death occurs between blastula and pluteus stages. When applied to mammalian spleen cells which have been induced to form antibody, the compound is inhibitory only during early stages of synthesis of antibody when ability to produce specific protein is increasing (8). Once maximal synthetic levels are reached, no inhibition results from BUDR treatment. These data can best be interpreted in the light of Makela and Nossal's experiments (21, 23) which suggest that cell division comes to a halt as antibody production commences and before it has reached maximal level. Thus, if BUDR is available to be incorporated while division is still occurring, subsequent antibody titers for the whole cell population will be abnormally low—presumably because part of the population contains abnormal DNA. We propose that the same general reasoning applies to the pancreas system.

These interpretations assume the absence of visualizable zymogen granules to indicate "non-differentiation." The actual block to granulogenesis is, of course, not defined. We do not know whether specific protein which ultimately is included in the granule cannot be synthesized with abnormal DNA present or whether the packaging of protein into granules is prevented. With these reservations in mind, the hypothesis can be advanced that only cells not synthesizing DNA differentiate normally in the presence of BUDR. It is interesting that BUDR treatment just prior to cessation of DNA synthesis, as indicated by thymidine incorporation, results in complete blockage of granule formation. This suggests that unaltered DNA functions in a differentiative sense in pancreatic acinar cells close to the time when DNA synthetic cycles cease and specialized synthesis begins. The latter is indicated by ultrastructural data (16) and by increase in pancreatic amylase activity (28). In turn, this suggests that sufficient accumulation of DNA-derived information (messenger RNA?) does not occur during the inductive period (0 to 36 hours *in vitro*), or even beyond, before a non-dividing population is set up (*ca.* 36 to 60 hours). These considerations emphasize our ignorance about the early form of stabilization of the epithelium suggested by the mesenchyme removal experiments. The mechanism of this earlier stabilization, obviously closely related to the *modus operandi* of the inducing influence, remains unknown, but clearly is a chief target for further investigations.

Finally, we wish to turn to a rather general point concerning the origin of pancreatic acini. In contrast to most textbook descriptions, none of our observations on embryonic pancreas suggest that the duct is involved in production of acini. Instead, the epithelial periphery—presumably a direct descendant of the initial evagination of the gut wall—is the region of thymidine incorporation, cell proliferation, and, presumably, origin of acini. Duct cells in freshly excised pancreas of varying ages, or in the centers of epithelial explants, incorporate H^3TDR at a much lower rate than do the peripheral pre-exocrine cells.

A similar low rate is found in islet cells. This common characteristic of duct and islet cells may suggest a similarity in origin, a similarity which is already implied by a number of experiments which indicate that duct can produce abundant islet tissue after experimental manipulation (see Bensley (1) for early data; see also 5, 15). Furthermore, Fitzgerald's recent investigations (9) cast doubt on the role of duct in production of new acinar cells during recovery from ethionine treatment.

Our observations show that few or no peripheral cells of the pancreas *in vivo* incorporate H^3TDR by the 18th day of development. Thus, the main exocrine proliferative population has apparently disappeared as a consequence of its differentiation. Any further increase in acinar cell number under normal or regeneration conditions may result from infrequent mitoses in acini or ducts (15, 26). It is clear that precise data are needed on the state of differentiation of such dividing cells at the time when their DNA is replicated.

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REFERENCES

1. Bensley, R. R., Structure and relationships of the Islets of Langerhans, *in* The Harvey Lectures, 1914–1915, **250,** Philadelphia, J. B. Lippincott Co.
2. Berrill, N. J., Cell division and differentiation in asexual and sexual development, *J. Morphol.*, 1935, **57,** 353.
3. Bond, V. P., Fliedner, T. M., Cronkite, E. P., Rubini, J. R., and Robertson, J. S., Cell turnover in blood and blood-forming tissues studied with tritiated thymidine, *in* The Kinetics of Cellular Proliferation, (F. Stohlman, Jr., editor), 1959, **188,** New York, Grune and Stratton.
4. Cheong, L., Rich, M. A., and Eidinoff, M. L., Introduction of 5-halogenated uracil moiety into deoxyribonucleic acid of mammalian cells in culture, *J. Biol. Chem.*, 1960, **235,** 1441.
5. Coupland, R. E., The survival and growth of pancreatic tissue in the anterior chamber of the eye of the albino rat, *J. Endocrinol.*, 1960, **20,** 69.
6. Dawson, A. B., Cell division in relation to differentiation, *in* Second Symposium on Growth and Development, *Growth*, suppl., 1940, 91.
7. Djordjevic, B., and Szybalski, W., Genetics of human cell lines. III. Incorporation of 5-bromo and 5-iododeoxyuridine into the deoxyribonucleic acid of human cells and its effect on radiation sensitivity, *J. Exp. Med.*, 1960, **112,** 509.
8. Dutton, R. W., Dutton, A. H., and Vaughn, J. H., Effect of 5-bromouracil deoxyriboside on the synthesis of antibody *in vitro*, *Biochem. J.*, 1960, **75,** 230.
9. Fitzgerald, P. J., The problem of the precursor cell of regenerating pancreatic acinar epithelium, *Lab. Invest.*, 1960, **9,** 67.
10. Fujita, S., Kinetics of cell proliferation, *Exp. Cell Research*, 1962, **28,** 52.
11. Golosow, N., and Grobstein, C., Epitheliomesenchymal interaction in pancreatic morphogenesis, *Develop. Biol.*, 1962, **4,** 242.
12. Grobstein, C., Differentiation of vertebrate cells, *in* The Cell (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, Inc., 1959, **1,** 437.
13. Grobstein, C., Interactive processes in cytodifferentiation, *J. Cell. and Comp. Physiol.*, suppl. 1, 1962, **60,** 35.
14. Hakala, M. T., Mode of action of 5-bromodeoxyuridine on mammalian cells in culture, *J. Biol. Chem.*, 1959, **234,** 3072.
15. Hughes, H., An experimental study of regeneration in the Islets of Langerhans with reference to the theory of balance, *Acta Anat.*, 1956, **27, 1.**
16. Kallman, F., and Grobstein, C., Fine structure of differentiating mouse pancreatic exocrine cells in culture, *J. Cell Biol.*, 1964, **20,** 399.
17. Karnofsky, D. A., and Basch, R. S., Effects of 5-fluorodeoxyuridine and related halogenated pyrimidines on the sand dollar embryo, *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 61.
18. Kit, S., Beck, C., Graham, O. L., and Gross, A., Effect of 5-bromo-deoxyuridine on deoxyribonucleic acid-thymine synthesis and cell metabolism of lymphatic tissue and tumors, *Cancer Research*, 1958, **18,** 598.
19. Koch, W. E., and Grobstein, C., Transmission of radioisotopically labeled materials during embryonic induction *in vitro*, *Develop. Biol.*, 1963, **7,** 303.

20. LAJTHA, L. G., On DNA labeling in the study of the dynamics of bone marrow cell populations, *in* the Kinetics of Cellular Proliferation, (F. Stohlman, Jr., editor), 1959, **173,** New York, Grune and Stratton.
21. MAKELA, O., and NOSSAL, G. J. V., Autoradiographic studies on the immune response. II. DNA synthesis amongst single antibody-producing cells, *J. Exp. Med.*, 1962, **115,** 231.
22. MONESI, V., Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine, *J. Cell. Biol.*, 1962, **14,** 1.
23. NOSSAL, G. J. V., and MAKELA, O., Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation, *J. Exp. Med.*, 1962, **115,** 209.
24. PEARSE, A. G. E., Histochemistry, Theoretical and Applied, Boston, Little, Brown and Co., 2nd edition, 1961, 916.
25. PELC, S. R., On the question of renewal of differentiated cells, *Exp. Cell Research*, 1963, **29,** 194.
26. POLLISTER, A. W., Notes on cell division in the pancreas of the dogfish, *Anat. Rec.*, 1930, **44,** 29.
27. QUASTLER, H., Some aspects of cell population kinetics, *in* The Kinetics of Cellular Proliferation, (F. Stohlman, Jr., editor), New York, Grune and Stratton, 1959, 218.
28. RUTTER, W. J., WESSELLS, N. K., and GROBSTEIN, C., Control of Specific synthesis in the developing pancreas, *J. Nat. Cancer Inst.*, 1964, in press.
29. SCHOOLEY, J. C., Autoradiographic observations of plasma cell formation, *J. Immunol.*, 1961, **86,** 331.
30. SIDMAN, R. L., MIALE, I., and FEDER, N., Cell proliferation and migration in the primitive ependymal zone; an autoradiographic study of histogenesis in the nervous system, *Exp. Neurol.*, 1959, **1,** 322.
31. SIDMAN, R. L., Histogenesis of mouse retina studied with thymidine H3, *in* The Structure of the Eye, (G. K. Smelser, editor), New York, Academic Press, Inc., 1961, 487.
32. STOCKDALE, F. E., and HOLTZER, H., DNA synthesis and myogenesis, *Exp. Cell Research*, 1961, **24,** 508.
33. STOCKDALE, F. E., HOLTZER, H., NAMEROFF, M., and MADDEN, J., Inhibition of myogenesis by bromodeoxyuridine *in vitro,* American Society for Cell Biology, Abstracts, 2nd Annual meeting, 1962, 177.
34. STOHLMAN, F., JR., editor, The Kinetics of Cellular Proliferation, New York, Grune and Stratton, 1959.
35. WAYMOUTH, C., Nature of the stimulus to mitosis, *in* The Mitotic Cycle, (A. Hughes, editor), New York, Academic Press, Inc., 1952, 163.
36. WEISS, P., Principles of Development, New York, Henry Holt and Co., 1939.
37. WESSELLS, N. K., Effects of extra-epithelial factors on the incorporation of thymidine by embryonic epidermis, *Exp. Cell Research*, 1963, **30,** 36.
38. YOUNG, R. W., Cell proliferation and specialization during endochrondral osteogenesis in young rats, *J. Cell Biol.*, 1962, **14,** 357.
39. ZAMENHOFF, S., and GRIBOFF, G., *E. coli* containing 5-bromouracil in its deoxyribonucleic acid, *Nature*, 1954, **174,** 307.
40. ZAMENHOFF, S., REIMER, B., DEGIOVANNI, R., and RICH, K., Introduction of unnatural pyrimidines into deoxyribonucleic acid of *Escherichia coli*, *J. Biol. Chem.*, 1956, **219,** 165.