14 February 1964, Volume 143, Number 3607 SCIENCE

Cytodifferentiation and Its Controls

Intrinsic and extrinsic cellular controls intimately interact in differentiative synthesis.

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Diversification of cell types during the course of development is widely regarded as one of the most enigmatic and challenging problems of modern biology. Recognition that this is a crucial problem is not new. Recent years, however, have been marked by a sense of urgency and expectancy—as though great things were just over the horizon. Not only do long-time students of the subject feel this but so, too, do investigators in other areas who, increasingly, have been looking over the fence, quietly surveying the terrain, and regrouping their forces for entry into what seem to be very green pastures.

The source of the excitement, of course, is the increased familiarity with the interior of the cell resulting from joint use of the electron microscope, the ultracentrifuge, and the fraction collector. Coupled with the high-resolution techniques of microbial genetics, these studies have yielded models of the cell and its controls which seem to require only a properly placed switch or two to allow for the formation of an erythrocyte, or a liver cell, from an undistinguished zygote. That no one has yet seen such simple switching occur with cells rather than models may be mere happenstance. It may, however, indicate the need for some caution in approaching these promising green pastures.

My purpose is to describe progress made by my colleagues and me along a path chosen to exploit the new knowledge of neighboring fields while preserving the fundamental orientation of the traditional developmentalist. In describing this path I will be asserting my conviction that the approach it represents is an important and useful one to the problem of differentiation. I support this contention by outlining our particular methods and results against the background of some general statements about the problem as I see it. I hope I will not be misinterpreted: No one should think I believe there are no alternative approaches as likely, or even more likely, to yield important new insights.

Developmental diversification of cell types, to which the term *cytodifferentiation* is applied, is part of the larger phenomenon of differentiation which embryologists identified at the turn of the century as a major component of development. Development itself is progression of the organism in its life history, most frequently from the relatively small and simple to the relatively large and complex. Small-to-large is the growth component of development; simple-to-complex is the order-increasing component, to which various names have been applied. Whatever it is called, it includes increasing heterogeneity as well as advance to higher levels of order. Increasing heterogeneity is manifested as increase in kinds of things in increasing numbers of compartments—new kinds of molecules, organelles, cells, tissues, and so on. Increases in the number of kinds of cells, and the processes by which cells of more general properties in earlier stages change into cells of more special properties in later stages, are what we refer to as cytodifferentiation.

Thus, in multicellular organisms, differentiating cells are parts of organisms, and as they differentiate they behave increasingly as specialized components rather than as wholes. The differentiations of individual cells are linked in a pattern which "makes sense" at the level of the whole. It is, therefore, not surprising that controlling influences are exerted by sources extrinsic to the individual cells. Nonetheless, cells in isolation also can behave as wholes, and thus we know that cells have their own inner integrating systems. In these terms, a central problem of cytodifferentiation is the identification and characterization of control factors extrinsic to the cell and the elucidation of the mechanisms by which these impinge upon the cell's inner controls.

Defining an Experimental System

It follows that one kind of experimental system which is useful in the study of cytodifferentiation is a system in which cells are differentiating in patterns which "make sense" in terms of the phenomenon of the next higher order. In such systems individual cells may be expected to be subject to normal mechanisms of extrinsic control. The system should be isolated from effects of phenomena of a still higher order, since these may complicate both manipulation and interpretation. This suggests, as subject, either a relatively simple organism or an iso-

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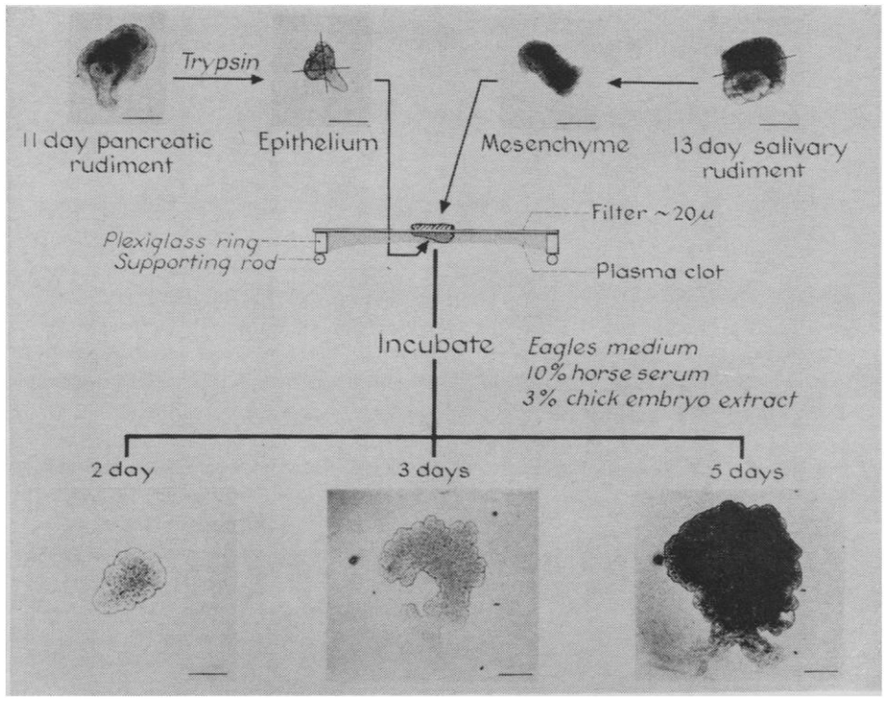

Fig. 1. Technique for preparing standard transfilter cultures of pancreatic epithelium and salivary mesenchyme. Intact rudiments may be cultured either "in the clot" in the position of the epithelium, or "on the platform" in the position of the mesenchyme.

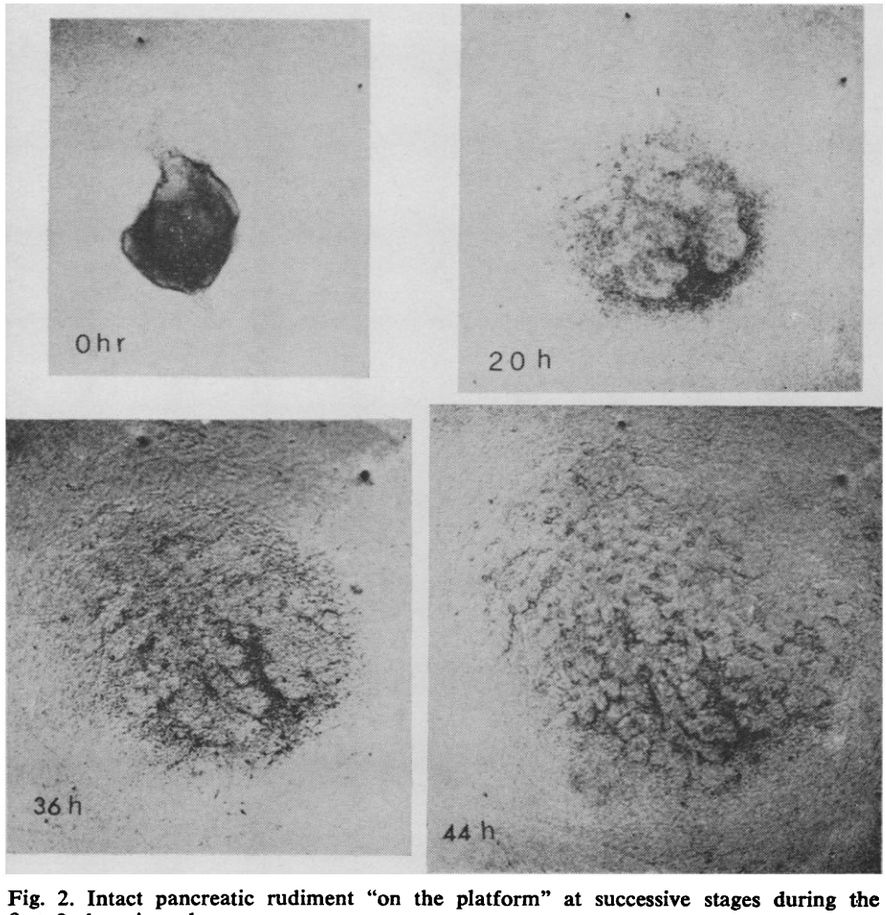

Fig. 2. Intact pancreatic rudiment "on the platform" at successive stages during the first 2 days in culture.

lated portion of a more complex one. The components of the system should be separable, and it should be possible, after separation, either to keep them in isolation or to recombine them to test for componential interactions. The differentiative behavior of individual cells should be readily, and preferably quantifiably, characterizable. Finally, the cells should lend themselves operationally both to the kinds of manipulation and observation which have proved useful in examining intercellular relations and to the kinds which have yielded insight into intracellular controls.

No experimental system is likely to present the perfect answer to these requirements. However, the developing pancreatic rudiment of the mouse is promising for several reasons. The adult pancreas has been the subject of intensive cytological, physiological, and biochemical study (*1*) and is a familiar object from these points of view. It has a definite but relatively simple architecture, with at least two specialized cell types—acinar and islet. The first produces a number of well-characterized enzymes; the second, two well-characterized hormones. The cells, particularly the exocrine acinar cell, are among those whose study has provided the basis for present conceptions of intracellular regulation of biosynthesis. Moreover, the mouse—genetically the best known of the vertebrates—offers opportunity for controlled variation of the genetic parameter.

Accordingly, several years ago Golosow and I made some preliminary developmental studies in vitro on the mouse pancreas (*2*). We began by confirming earlier findings (see *2*) that cultured embryonic pancreas continues its development and differentiation. The culture techniques were those employed earlier in studies of kidney, salivary-gland, somites, and other rudiments of mouse embryos (*3*). These are modifications of classical procedures for organ culture, in which advantage is taken of newer materials and procedures. Highly porous, thin membrane filters, mounted on Plexiglas rings, as indicated in Fig. 1, provide the substrate for culturing and allow exchange with the nutrient medium below. The nutrient (changed daily) is the basal medium of Eagle (*4*) supplemented with horse serum (10 percent) and chick embryo extract (3 percent). The incorporation of penicillin, streptomycin, and Mycostatin makes it possible to

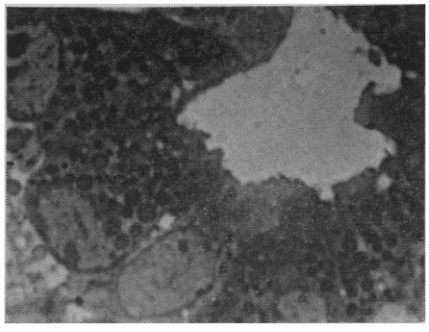

Fig. 3. Section of methacrylate-embedded and stained pancreatic epithelium after 5 days of culture, as viewed with the phase-contrast microscope. Note the prominent zymogen granules in many of the cells, arranged around acinar lumina.

culture the cells in loosely covered glass dishes in an incubator at high humidity, in air containing 5 percent carbon dioxide. A dorsal pancreatic rudiment dissected from an 11-day mouse embryo continues its development when placed on the membrane platform under these conditions (Fig. 2). The original, relatively simple, epithelial component grows and ramifies to form many acini, which become opaque on the fourth or fifth day. A section through such a culture on the fifth day (Fig. 3) shows that many of the acinar cells are packed with eosinophilic zymogen granules, presumably packets of stored enzyme awaiting secretion. In addition, one can identify nonsecretory ducts and, very occasionally, packets of cells resembling islets—the source of insulin and glucagon in the normal pancreas.

Pancreatic Exocrine Differentiation

Differentiation of the acinar exocrine cells is detectable with the optical microscope on the fourth to fifth day of culture, but it can be detected considerably earlier by other methods. Frances Kallman (*5*) examined the differentiating acinar cells of the rudiments, both in the embryo and in vitro, with the electron microscope. The zymogen granules visible with the optical microscope on the fifth day of culture (corresponding to the 16th day of embryonic life) appear in the electron micrographs as obvious opaque bodies. On the fourth day in culture precursor prozymogen granules are equally obvious. However, even on the third day some cells can be identified as probably embarked on a differentiative course. Although prozymogen granules are seen infrequently in these cells and are quite small, the endoplasmic reticulum is clearly modified, for many of the ribosome-studded membrane profiles are expanded into cisternae. The Golgi material at this stage also suggests activity, as indicated by an increase in the number of profiles and vesicles of various size. The ultrastructural picture is the one to be expected if product synthesis has already begun, and if material is accumulating in the endoplasmic reticulum and beginning to be transformed in the Golgi zone into secretion granules. A day earlier, on the 13th day of embryonic development or the end of the second day of culture, the cytoplasm is packed with ribosomes, but the number of endoplasmic reticular profiles is relatively small, and only occasional and small, expanded cisternae are seen. It is interesting to note that at this stage and earlier, when there is little evidence of accumulation of secretory material, the abundant ribosomes are characteristically in rosettes, a configuration reminiscent of configurations described for message-reading, by the ribosomes, from associated strands of RNA (*6*).

The ultrastructural indication that accumulation of specialized product is well under way by the third day is in accord with studies of the amylase content of maturing rudiments in vivo and in vitro carried out by Rutter (*7*). Specific amylase activity increases by approximately five orders of magnitude during the period of culture, and this increase is only slightly less than that observed in the normally developing rudiment (Fig. 4). The amylase activity by the third day of culture is 100 times the initial level on the first day. The inflection point of the curve in Fig. 4 is at about the end of the second day, a finding which corresponds well with the ultrastructural observations.

On the basis of these data, can we

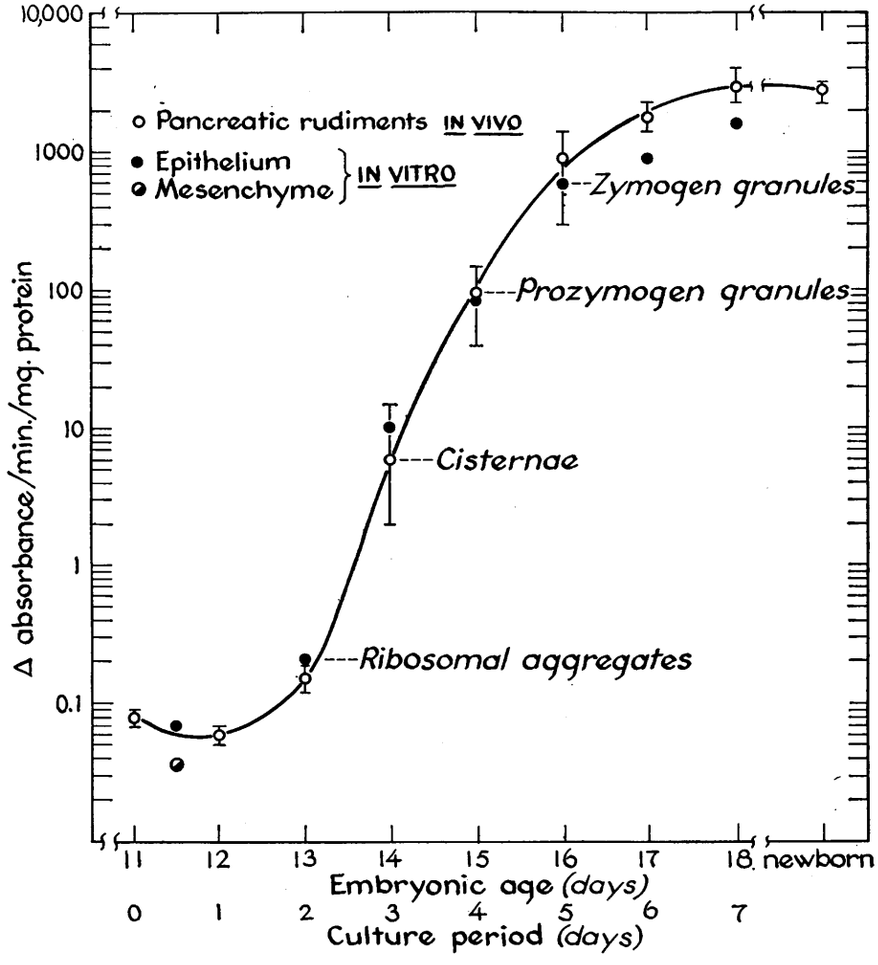

Fig. 4. Plot of amylase activity in the developing pancreas in vivo and in vitro. Characteristic ultrastructure is indicated at approximate stages along the curve.

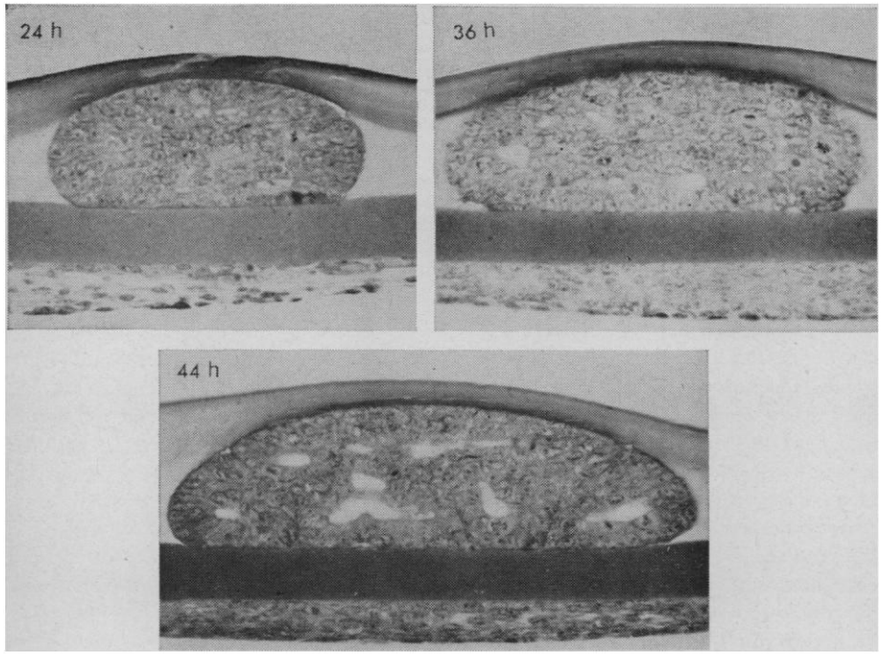

Fig. 5. Stained sections of pancreatic epithelium fixed at indicated times of culture. Note the growth in size and the increased orientation of cells around acinar cavities.

say when pancreatic acinar differentiation begins? With the optical microscope and ordinary methods of sectioning, exocrine differentiation is first identified on the fourth to fifth day, when frank zymogen granules can be clearly discerned. However, thinner sections embedded in methacrylate resin, when viewed with the phase-contrast microscope, show droplet-containing cells as early as the third day of culture. As has been noted, the electron micrographs show this period to be one of expanding cisternae and enlarging Golgi vesicles. The electron micrographs and the results of enzymatic assay are in accord in suggesting that some specialized synthesis occurs even earlier, perhaps after only 48 hours of culture. If differentiation is regarded as accumulation of specialized product, detection of its beginnings obviously may be limited by the method of assay. On the basis of the cited criteria of ultrastructure and amylase activity, one would say that acinar cells begin differentiating by the end of the second day. Conceivably, however, a more discriminating test or an assay for another enzymatic product—for example, trypsin, chymotrypsin, or ribonuclease—would show specialized synthesis even earlier for assay than amylase does. Parenthetically, one of the interesting questions to raise is: What is the degree of coupling in the synthesis of the several enzymatic products of the pancreatic acinar cell? For the moment, all we can say about the beginning of differentiation—as measured by specific synthesis—is that significant increase of amylase cannot be detected earlier than the end of the second day.

Criteria of Differentiation

But is synthesis, or accumulation, of specialized product the most appropriate measure of differentiation? Are there other criteria by which a differentiative state may be recognized? Are there differentiative changes which precede the earliest appearance of specialized product? These are old questions which have yet to be fully answered, and which lie close to the heart of the problem of differentiative mechanisms. In the case of the pancreas the following observations are relevant. As I have said, epithelial differentiation proceeds in the intact rudiment in vitro. It can be shown that an essential contribution to epithelial differentiation is provided by the surrounding mesenchyme (*2*). This is demonstrated by exposing the rudiment briefly to a solution of crude trypsin, making possible the separation of epithelial and mesenchymal components. The epithelial component, when clotted on the under surface of a filter assembly, spreads as a sheet but entirely fails to differentiate secretory acinar cells. If it is recombined with mesenchyme, however, either directly or across the thin membrane filter, epithelial differentiation occurs. Similar dependence of epithelial differentiation on mesenchyme has been shown for the salivary-gland, thyroid, kidney, skin, thymus, and other rudiments (*8*). In other words, epithelial differentiation, including that of the pancreas, is at least partly under extrinsic control—by a tissue of another type. This kind of heterotypic dependency of differentiation has long been known as embryonic induction. In the case of the pancreas the inducer clearly is of such nature that it can cross a 20-micron interspace provided by the thin, porous, membrane filter. I discuss more of this later. For the moment, the important point is that the trans-filter induction system makes it possible to interrupt the interaction at any time simply by removing the mesenchyme from the upper or platform surface of the filter. When this is done after increasingly long intervals of culture (*9*) it is found that removal prior to the 30th hour is not followed by acinar differentiation. Removal of the mesenchyme at the 30th hour allows minimal differentiation in some cultures, and removal of the mesenchyme at the 48th hour or later allows zymogen synthesis to occur in all cultures, the degree of synthesis increasing with the period of culture.

What is relevant to the question under discussion is the fact that 18 hours prior to the time when enzymatic or ultrastructural signs of differentiative product are discerned, changes occur which render at least some cells of the epithelium capable of differentiation in the absence of an inductive stimulus that they required earlier. From approximately the 30th to approximately the 48th hour of culture the epithelium shows none of the usual signs of definitive pancreatic differentiation. Yet a critical change has occurred, due to the influence of the mesenchyme, and only time seems to be needed for the effect to be manifested. This kind of change of properties, undetectable in terms of the criteria of the final state, has been demonstrated in many developmental pathways and has been variously referred to as determination, chemodifferentiation, or covert differentiation (*10*). The mechanisms of this covert state have yet to be elucidated, and it seems likely that in them may lie the crux of the puzzle of differentiation. What are the possibilities concerning the nature of this state as we now visualize them?

Nature of the Covert Phase

First, it is possible that we are dealing with an artifact of observation, that the covert phase is a period when synthesis of specific product is subliminal at resolutions presently attainable, and that with improved methods of detection the covert phase will disappear. Second, covertness may represent latency in the sense of preparation—a shifting of the cell "gears" to initiate new synthesis. It may represent the time, for example, necessary for the inducer to move into the cell, derepress certain structural genes, and allow their translated message to reach sites of protein synthesis in the cytoplasm. According to this view, the covert phase is different in nature from the overt phase but part of a continuous time course: the one is prelude to the other, but not otherwise separable from it.

A third possibility has long been entertained: that the covert phase is in fact the fundamental phase, differing not only in nature from the overt one but entirely separable from it and able to persist for long periods without overt expression. Whatever the mechanism, this possibility requires the assumptions that the covert phase begins with the essential event of differentiation, that some main-line switch closes, and that differentiated type is thereby determined. According to this view, all that follows this essential event is relatively inconsequential detail or realization, and "true differentiation" is covert with respect to the criteria applicable to the final state, stable from the outset, and presumably propagable, since cells frequently continue to divide during the covert phase. It is to be noted that this possibility, as compared with the first two, requires a different definition of differentiation, at least for the moment —one in terms of operations that determine what cells will do later on, not what they will do immediately after the essential event. It says that two populations now operationally indistinguishable must be different because they become distinguishably different later, even though they are kept under identical conditions.

The importance of resolving these alternative explanations of the covert phase lends special interest to the events occurring in cultured pancreas between about the 30th hour and the end of the second day of culture. One question to be asked is whether cells become "fixed" in their differentiative behavior at the beginning of the phase (as suggested by the third possibility). This question has been asked in connection with other developing systems (*11*), and the answer has usually been in the negative. Wessells and I undertook to answer it for the pancreas, in the following way (*9*). Epithelium which has been cultured from mesenchyme, across a membrane filter, for 30 or 40 hours can be removed from its clot with trypsin and recultured by reclotting in a new assembly. There is a clear difference in the behavior of the epithelium cultured for 30 hours and that cultured for 40 hours: the 30-hour epithelium rarely forms detectable zymogen in a total culture interval of 5 days, while the 40-hour epithelium invariably does so. However, if the 40-hour epithelium is cut into fragments before reculturing, its differentiative behavior varies, depending upon the size and disposition of the fragments. If it is cut into eight fragments of equal size and if these are combined in a close cluster so as to re-fuse into a single mass, the new culture always forms zymogen, though in somewhat reduced amount relative to the original unfragmented culture. Fragments of this size cultured individually never produce zymogen, even when all fragments of a particular epithelial mass are tested. This finding indicates that some cells which were covertly differentiated fail, later, to differentiate overtly if they are components of small fragments, whereas they do differentiate overtly if they are components of a larger mass.

This kind of experiment has obvious shortcomings as a means of deciding what is going on in particular cells from the 30th to the 40th hour of culture. Since, however, the test for covert differentiation reveals that the size of the cell population tested is an important parameter, one cannot exclude interactions among the epithelial cells themselves as possible contributors to the altered behavior between 30 and 40 hours. Indeed, when one compares the epithelium after culture from mesenchyme for 1 and 2 days, across a filter (Fig. 5), it is obvious that both the increased size of the population and the altered arrangement of the cells provide ample basis for changes in the interactions among the cells.

Changes in the orientation and shape of cells and in the homotypic relations of cells to one another and to their substrate have been emphasized as preliminaries to cytodifferentiation. In a number of developing systems (see *10*, *12*), interruption or inhibition of these changes has been correlated with failure of differentiation, though the causal connection is not clear. It is possible that the covert phase is at least partly an expression of such changes, and that differentiation as defined by the more usual criteria is their sequel. A number of investigators have suggested that the findings implicate the cell boundary—the surface membrane of the cell and immediately associated materials—in the initiation of differentiation (*13*). In particular it is suggested that boundary materials, especially when shared among cells, may play an important relay and transducing role in the transmission of extrinsic cues of higher-order integrative patterns (*14*). Definitive evidence, however, is still lacking about the nature of the boundary materials involved and the mechanism by which such changes may influence processes deeper in the cell.

DNA and Zymogen Synthesis

Let us set aside for the moment the question of the earliest steps in differentiation. There is evidence that, by the beginning of the third day of culture, important changes in properties relating to subsequent differentiation occur in pancreatic epithelium. Wessells (*15*) has gotten some idea of what is going on by observing the incorporation of tritiated thymidine, a process which reveals patterns of synthesis of DNA within the cell mass. By the 48th hour it is clear that the number of cells engaged in such synthesis is greater at the periphery of the tissue than at the center, and by the 72nd hour cells synthesizing DNA are largely confined to the superficial layer of the explant (Fig. 6). Labeling, followed by re-incubation for various periods, provides grounds for making the following statements with some assurance. By the 48th hour pancreatic epithelium under these culture conditions has a peripheral, germinal cortex, within which a high proportion of the outermost cells are in the DNA-synthesis phase of the mitotic cycle. These cells undergo actual mitosis in the immediately subperipheral zone—that is, lower in the cortex. Mitotic figures in this region are unlabeled in tissues fixed immediately after the incorporation of tritiated thymidine but are 100 percent labeled 7 hours later. The mitotic cells are, therefore, a later

phase of the peripherally labeling ones —that is, cells or nuclei move toward the center to divide. Some cells produced by the cortical proliferation accumulate toward the center, making up a postmitotic compartment of the population which enlarges during the next several days at the expense of the proliferative compartment. There is thus a periphero-central gradient of increasing age of maturation, and this is reflected in a centro-peripheral appearance of zymogen granules in the differentiating cells.

The findings provide clear indication of a generally reciprocal relation between DNA synthesis and zymogen synthesis, in accord with the long-held view that overt differentiation and mitotic activity tend to exclude each other (*10*, *16*). It should be noted, however, that there are complications, that not everyone accepts this view (*17*), and that there is need for particular caution in extending histological and cytological generalization to the molecular level—that is, to the question of the relation between replicative and transcriptional activity of DNA. Methods for approaching this latter problem are only now becoming available.

Wessells has been investigating the relation of nucleic acid synthesis to the formation of zymogen granules by treating the differentiating pancreatic epithelium with metabolic inhibitors for short periods at various times during culture. It is of considerable interest that the sensitivity of granule formation to actinomycin changes between the 48th and 72nd hour of culture. At actinomycin levels which fail to produce indication of general toxicity, the appearance of zymogen granules is inhibited by treatment prior to 72 hours, but appearance of the granules is not inhibited, in at least some cells, by treatment at 72 hours or later. The cells in which there is no inhibition at 72 hours tend to be centrally located and presumably represent the postmitotic "avant-garde" cells characterized by failure to incorporate thymidine. Rutter has made amylase determinations on the actinomycin-treated cultures and finds that treatment at 96 hours has little effect on amylase activity measured on the 5th day. Treatment at 72 hours, however, clearly reduces the amylase activity at the 5th day, and treatment at times earlier than the 72nd hour progressively further reduces the activity until, with treatment at 24 hours, the activity is virtually eliminated. Similarly, Wessells has found the 5-bromodeoxyuridine blocks the appearance of zymogen granules when administered prior to the 72nd hour, but not when administered at this hour or later. Among the more interesting explanations of these data is the possibility that "avant-garde" cells at the 72nd hour have already transmitted messenger RNA to their ribosomes and are therefore not affected in their specialized synthesis by actinomycin binding to their DNA. Continued experimentation with metabolic inhibitors, in conjunction with autoradiography, electron microscopy, enzyme assay, and chemical fractionation of the differentiating cultures, should yield definitive tests of this hypothesis, as well as insight into the initiating mechanisms for specialized protein synthesis in pancreatic acinar cells.

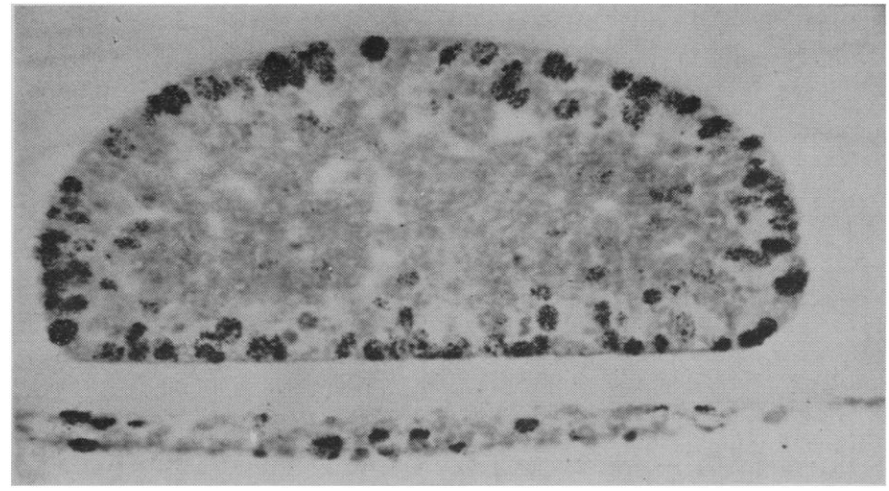

Fig. 6. Autoradiogram of transfilter culture labeled with tritiated thymidine. Note the localized incorporation primarily in the peripheral cells, with a central nonlabeling population.

The Input Leg and Differentiation

It is worth asking, however, whether the elucidation of these mechanisms of the output leg, from DNA to specialized product, will yield full comprehension of cytodifferentiation. Beyond question, it will represent considerable advance, particularly in showing that the intimate interplay of chromosome and cytoplasm which is fundamental to metabolic regulation in microorganisms also obtains in higher organisms. But there will remain the question of the input leg (*18*), the path of regulative impingement on the chromosome. This regulation is documented in a number of ways—not only for microorganisms but for higher organisms, and particularly for insects in the remarkable studies of the chironomids (*19*). We now know, for example, that an extrinsic agent—ecdysone—can turn chromosomal loci, in the functional sense, on and off, and that these functional loci are indeed those involved in genetic continuity and recombinations. It should be emphasized that this extrinsic control is exerted on *overtly differentiated* cells, at a stage corresponding to that of zymogen synthesis in pancreatic cells; what earlier steps *induced* the particular response pattern to ecdysone shown by salivary cells is still an open question.

I italicized *induced* in the preceding sentence to introduce my final point. The concept of induction and the concept of differentiation have been closely interlinked since the classic demonstrations of developmental dependency by Roux and Spemann. What these demonstrations say in modern terms is that the control systems of the cell in the development of higher organisms are subject to manipulation from without. To the extent that cytodifferentiation is controlled biosynthesis, the web of biosynthetic operations must be extrinsically regulable. If microbial regulation is a suitable model (*20*), we conclude that the complexity of higher-order controls in the multi-cellular system funnels down to a final common path on the chromosome. This conclusion implies two things worth noting. First, given the demonstrable complexity of higher-order controls, many factors may register in the final common path and operate as an embryonic

inducer. Second, an embryonic inducer need not be, and probably is not, identical in nature with the more direct —conceivably final-path—inducer of microbial systems.

The first point is particularly pertinent in view of the multiplicity of inducers which has, indeed, been found in primary induction (*21*). After the excitement of 30 years ago when the neuralizing action of killed tissues on gastrula ectoderm was discovered, there was a period of embarrassment of riches when everything "induced," culminating in the frustration of "self-induction." In recent years, renewed respect for the role and complexity of the responding tissue—an entirely healthy development—has led in some quarters to complete deprecation of the role of the inducer. Despite the dethronement of inducers from the status of "organizer substance" and the probability that they operate in very different ways in different instances, the nature of physiologically effective inducers remains an important potential clue to the character not only of embryonic integration in general but of differentiative controls in particular. The problem, however, is probably best approached not by testing the effect of various materials on an isolated responding system but by attempting to determine the nature, and the requisite conditions for transfer, of inductive materials in an intact, physiologically functioning interaction. In our laboratory at Stanford, my associates and I have been attempting to do this in several systems; here I mention only some recent results obtained by Rutter with the pancreas (*7*).

As I said earlier, pancreatic epithelium, under the conditions of our experiments, continues its differentiation only in the presence of mesenchyme. On finding that this requirement could be met not only by pancreatic mesenchyme but by mesenchyme from any source tested, it seemed possible that the responsible materials might be fairly generally distributed, and hence abundant enough to be chemically isolated. When chick embryo mesenchyme was found to be active, it appeared that chick embryos might be a suitably abundant source. The nutrient medium, of course, contains embryo juice, at the 3 percent level, so the effect of an increase in the concentration of embryo juice was tested. At concentrations of 10 to 20 percent, growth and differentiation of pancreatic epithelial cells occurred in the absence of mesenchyme, though the amount of zymogen produced did not seem as high as the amount produced with a control medium containing mesenchyme and embryo juice in 3-percent concentration. Raising the concentration of embryo juice still further, to 40 percent, did not increase the response—in fact, there was evidence of toxicity and inhibition, and deleting horse serum from the medium, or raising the concentration of horse serum above the normal 10 percent, similarly inhibited acinar differentiation. It seemed clear that in this experimental system, several factors could act as differentials. In particular, something in chick embryo juice at proper concentration was effective in the absence of mesenchyme.

We do not yet know the specific nature of the active material or materials, but certain properties of the active fraction have been defined. There is no activity in chick embryo juice ultrafiltrate, nor in the soluble fraction after sedimentation at 100,000*g*. There is activity in the sediment obtained at 1000*g* and 10,000*g*, and activity is high in the sediment obtained at 100,000*g*. The activity of the sediment declines in a few hours at 37°C and is eliminated by exposure to trypsin. Treatment with RNA-ase and DNA-ase has little effect. No comparable effects are exerted by adult liver microsomes, by mitochondria, or by collagen, DNA, and RNA preparations tested in a wide range of concentrations.

It is too early to interpret these results, especially to specify the relation of the active material of embryo juice to the physiologically active product of mesenchyme. Nonetheless, it is encouraging to find that the properties observed so far are in general conformity with the results of studies of induction of kidney tubules by dorsal spinal cord (*22*). In both instances the evidence implicates large-molecular materials which are sensitive to trypsin and so organized as probably to have low mobility under physiological conditions. In the case of the transfilter interaction of spinal cord and metanephrogenic mesenchyme, activity was significantly restricted when the average diameter of the filter pores was about 0.1 micron. It will be interesting to learn whether there is comparable restriction of the mesenchyme factor. Meanwhile, whatever the physiological significance of the embryo juice factor, it has the practical significance of providing a differentiating epithelial culture in the absence of mesenchyme. Tests are in progress to determine whether the material is a general promoter of differentiation in vitro. Salivary epithelium gives only a weak response to the particulate factor, and metanephrogenic and somitic mesenchyme are not induced by the particulate factor to form kidney tubules and cartilage, respectively, as they are by appropriate regions of embryonic spinal cord.

Cell and Organism

In conclusion, let us return to the theme with which we began. A powerful arsenal of techniques and knowledge recently has been made available to students of development by colleagues in related fields. Thus, it is justifiable to expect very rapid, early advances in our knowledge of cytodifferentiation. In particular, we may expect that differentiation at the cell level soon will be placed in proper perspective in the spectrum of cell responses interpretable in terms of a general model of intracellular control of biosynthesis. At the same time, as a phenomenon peculiarly characteristic of multicellularity, it will have to be related to the subtle cues which integrate a collection of cells into an organism. The mechanisms of short-range, homotypic interaction between cells of like type and of heterotypic interactions of conceivably somewhat longer range between cells of unlike type will have to be related to the intracellular control system. In the process, the successively more encompassing shells of interactive control will merge the general model of the cell into a general model of the organism. Progress will be faster if, in applying newer knowledge at the levels of the molecule and the cell, we keep in mind the organism and its controls in searching for a full conception of developmental diversification.

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Microenvironments and Mesoamerican Prehistory

Fine-scale ecological analysis clarifies the transition to settled life in pre-Columbian times.

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A crucial period in the story of the pre-Columbian cultures of the New World is the transition from a hunting-and-collecting way of life to effective village farming. We are now fairly certain that Mesoamerica (*1*) is the area in which this took place, and that the time span involved is from approximately 6500 to 1000 B.C., a period during which a kind of "incipient cultivation" based on a few domesticated plants, mainly maize, gradually supplemented and eventually replaced wild foods (*2*). Beginning probably about 1500 B.C., and definitely by 1000 B.C., villages with all of the signs of the settled arts, such as pottery and loom-weaving, appear throughout Mesoamerica, and the foundations of pre-Columbian civilization may be said to have been established.

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Much has been written about food-producing "revolutions" in both hemispheres. There is now good evidence both in the Near East and in Mesoamerica that food production was part of a relatively slow *evolution*, but there still remain several problems related to the process of settling down. For the New World, there are three questions which we would like to answer.

1) What factors favored the early development of food production in Mesoamerica as compared with other regions of this hemisphere?

2) What was the mode of life of the earlier hunting-and-collecting peoples in Mesoamerica, and in exactly what ways was it changed by the addition of cultivated plants?

3) When, where, and how did food production make it possible for the first truly sedentary villages to be established in Mesoamerica?

The first of these questions cannot be answered until botanists determine the habits and preferred habitats of the wild ancestors of maize, beans, and the various cucurbits which were domesticated. To answer the other questions, we must reconstruct the human-ecological situations which prevailed.

Some remarkably sophisticated, multidisciplinary projects have been and still are being carried out elsewhere in the world, aimed at reconstructing prehistoric human ecology. However, for the most part they have been concerned with the adaptations of past human communities to large-scale changes in the environment over very long periods—that is, to alterations in the *macroenvironment*, generally caused by climatic fluctuations. Such alterations include the shift from tundra to boreal conditions in northern Europe. Nevertheless, there has been a growing suspicion among prehistorians that macroenvironmental changes are insufficient as an explanation of the possible causes of food production and its effects (*3*), regardless of what has been written to the contrary.

Ethnography and Microenvironments

We have been impressed, in reading anthropologists' accounts of simple societies, with the fact that human communities, while in some senses limited by the macroenvironment—for instance, by deserts or by tropical forests (*4*)—usually exploit several or even a whole series of well-defined *microenvironments* in their quest for food (*5*). These microenvironments might be defined as smaller subdivisions of large ecological zones; examples are the immediate surroundings of the ancient archeological site itself, the bank of a