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THE DIFFERENTIATION OF CELLS

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The differentiation of cells, as I shall try to make clear in a moment, is one of the most fundamental and fascinating problems of biology. After a long history of ups and downs, extending over a period longer than the century of this Academy's existence, attempts to solve the problem now appear to be making remarkable progress. There is widespread and growing conviction among biologists that this will be the area of one of the next great triumphs of biology in the decades immediately ahead. As you will see, the progress already made provides some basis for this optimism. But first I shall explain the nature of the problem and of the current reorientation of research.

The Problem.—A cell consists of a nucleus surrounded by cytoplasm. The membrane-bounded nucleus contains, among other things, the chromosomes with their genes. The cytoplasm is also membrane-bounded and contains an ordered array of fluids, gels, granules, fibers, and membranes grouped into characteristic organelles.

Although life must have arisen and long evolved in forms simpler than a cell, every form of life now existing—with minor exceptions—is either a cell or, after having been a single cell at one stage of its life, came to be composed of many cells. The cell is the basic unit of structure and function in existing organisms.

The differentiation of cells is most familiarly associated with cell division. We are all aware, for example, that a human being starts life as a single cell, a fertilized egg. The egg divides into two cells, then each of these into two more, and so on through many successive cell divisions until the more than 10^{15} cellular building blocks of the human body have been formed. Even this is not the end, for cell divisions continue in certain organs and tissues throughout life. During development, the first cells to arise seem to be identical, but soon diversities appear. At first they are relatively slight and generalized, but later the cellular differences become greater and greater, yielding more and more specialized cell types. For example, nerve cells are specialized for transmission of signals, and muscle cells for contraction. Finally, about 100 different kinds of normal cells can be distinguished by their structure and function. Unfortunately, sometimes abnormal cells, such as tumor cells, also arise sooner or later. By differentiation of cells is meant in part this appearance of diversity, both normal and abnormal, among the cell progeny of the egg cell during the whole of the life of the individual. How this increase of heterogeneity occurs and how its marvelously precise and regular ordering in space and time are governed so as to yield at every stage an integrated functioning

multicellular individual are among the deepest and most challenging problems of biology.

In this most familiar example, cellular differentiation is associated with cell division and so might be imagined to depend ultimately upon the parceling out of diverse parts of one cell into different daughter cells. But it is important to recognize that cellular differentiation can also occur in the absence of cell division. This happens, for example, as cells grow older. Cellular changes also develop in response to changes in the milieu, including contact with another kind of cell or with its diffusing or circulating products such as hormones. Transformation of cells without division is dramatically evident in the life of a slime mold such as Dictyostelium which has long fascinated investigators. After a colony of separate creeping amoebae, all of which may be descended from a single ancestral amoeba by repeated divisions, has exhausted the available food supply (bacteria), the dispersed cells suddenly aggregate into a mass or slug. Without further cell division, the apparently identical amoebae of the slug pile up to form an upright stalk crowned by a capsule containing spores. Some amoebae have differentiated into stalk cells, some into capsule cells, some into spores—all very different from each other and from the amoebae of the slug.

Such integrated diversification without cell division among the identical cells of a multicellular unit is paralleled by the integrated diversification among the parts of a single cell. The unicellular animals include perhaps the most complexly organized of all cells. Yet some of them encyst in a very much simplified form and, when they excyst, recreate their amazingly complex but precise pattern of intracellular differentiation in an elegantly ordered series of developmental processes. Comparable precision and elegance mark the structural developments accompanying cell division. Two identical asymmetric patterns of complex structure are created from one without dedifferentiation, that is, while maintaining all the differentiated parts of the original cell (see Fig. 2). I shall come back to this highly sophisticated intracellular biological engineering later, for it reveals a neglected principle of cellular differentiation. At this point I wish only to stress that differentiation among cells is closely paralleled by differentiation within a cell. This parallelism and its important implications are too often ignored.

From whatever level we look at cell differentiation, we see a continuously graded series from the most stable, apparently irreversible, to the most transient. Although some students restrict the term "differentiation" to the extreme class of apparently irreversible changes, I have shown elsewhere[1] that there is no justification in nature for so narrow a view. Exactly the same cellular traits appear as irreversible or unalterable in some cells and as readily reversible or alterable in other closely related cells. It is not the change which varies in such cases, but associated mechanisms which result in its fixation or modifiability. The broad inclusive domain of cell differentiation then becomes virtually coextensive with cell physiology; it is a universal and all but continuous process in living nature.

Could such varied phenomena be explained on a single principle? If so, what is it? If not, are there only a few or many underlying principles? What are they? I believe already available knowledge justifies concluding that at least two basic principles are involved, and I shall discuss them. However, a single hypothesis is in fact guiding most of the current investigations, and there is a determined effort

to see how far one can go with this alone. The results already are spectacular. The dominant faith in this fruitful hypothesis stems from a recent reorientation of thought and research which will now be set forth.

The Current Reorientation.—The current reorientation is really quite simple, involving only a change in the basic assumption of the role played by the genes. Formerly, it was assumed that the whole set of genes was active in every cell. Hence, cells that have the same set of genes cannot become diverse by reason of direct genic action. There was indeed every reason to believe, and none to disbelieve, that (with minor and negligible exceptions) cells arising by ordinary division from a common ancestral cell had exactly the same set of genes. In the process of cell division, each chromosome replicates exactly, and one of the two identical daughter chromosomes passes to each daughter cell. Thus all the cells of the body, descended from the egg cell, would have the same set of chromosomes and genes. The cell differentiations arising during development therefore appear not to be due to possession of different genes. Nor, on the assumption that the genes were all performing the same primary actions in all these diverse cells, could the differentiations be due to direct genic actions. This view seemed to be reinforced by the occurrence of differentiation within single cells in the absence of cell division, allowing no possibility of change in the set of genes. Hence, it seemed useless to consider direct genic action as relevant to the problem of cell differentiation. So, attention was directed away from the genes to the cytoplasm and the cellular milieu. The milieu was often clearly changing and the cytoplasm obviously divided unequally in some cell divisions. These then became the focus of attention.

Yet there was a justifiable feeling of uneasiness. For the whole of development, including its many progressive steps of cell differentiation, was surely hereditary. And, in well-studied organisms like the fruit fly or corn, virtually every step in development was shown to be blockable or modifiable by genic mutations.

The paradox or dilemma was formally resolved by stressing correctly that what happens in a cell depends both on what genes are present and what cytoplasmic substrates are present for them to act upon. Change of either the genes or the cytoplasm changes the results. Normally, during development the genes remain the same, but the cytoplasm varies. Hence, it was argued, the cytoplasm, not the genes, is the decisive differential in cellular differentiation, and the genes may be safely ignored. This view long dominated thought and research in this field.

A very different view began to emerge about 25 years ago. One by one there began to accumulate evidences for active and inactive states of chromosomal material and for cells with identical sets of genes exhibiting "hereditary" difference for gene-controlled traits. For example, such indications of active and inactive genic states, some persistent and some transient, emerged repeatedly in my studies and those of my associates on the unicellular animal Paramecium beginning as far back as 1937 and coming strongly to the fore by 1948 with our investigations of the genetics of certain protein antigens.[1] Without attempting to give a full history of the course taken by this change of viewpoint, we may note at once that the most deeply analyzed and most influential study, which has become a classic of modern biology, is the one on genetic control of the production of the enzyme β-galactosidase in the bacterium *Escherichia coli*. Begun by Monod and brilliantly pursued by him, his collaborators (especially Jacob), and by many others, this great series

of investigations has provided and continues to provide model systems of the control of genic action.[2] As a result, it is now quite clear that a gene can be responsive to signals that regulate its degree of activity from complete inactivity, or almost so, to maximal activity. Of course, it is now known that activity means primarily production of complementary RNA (so-called genic messenger) and, through the messenger, of specific polypeptide.

These discoveries and models brought the genes back to the attack on the problem of cellular differentiation. Cellular differences, even among cells with identical sets of genes, could be due to the activity of different genes in those identical sets of genes. One no longer had to think of all the genes being active all the time. This is the essential reorientation of thought which guides most current research on cellular differentiation. It provides the now dominant hypothesis—one might almost say the principle or article of faith—that cellular differentiation is ultimately traceable to and due to variable gene activity.

Current Status of the Hypothesis of Variable Genic Activity.—When I reviewed[1] four years ago for this Academy the status of the hypothesis of variable genic activity as a basis of cell differentiation, it was necessary to cite evidence mainly from work on microorganisms and to argue for its generality largely on faith. Now, however, work on higher organisms has made such progress that the faith has been vindicated as the following account of representative examples will show.

One way to estimate genic activity is to examine the minute intracellular particles, the ribosomes, which are its sites. When genes are active, that is, when they are making their RNA messengers, the messengers become associated with ribosomes and the complex operates in polypeptide production. After extraction from cells, ribosomes which are not so engaged will become engaged if offered messenger RNA under appropriate conditions. This provides a general measure of genic activity. The degree to which extracted ribosomes will make polypeptides outside the cell when given messenger RNA is held to measure the degree to which they are *not* already so engaged inside the cell, that is, the degree to which the cell's genes were not active in making messenger RNA.

A number of embryologists have applied this sort of test at various stages in the development of embryos. For example, Nemer[3] has done so with the sea urchin. During the early cell divisions of the egg, before cell differentiations are determined, the ribosomes appear to be relatively inactive in polypeptide production. They are, however, perfectly competent to act, for when they are removed from the egg and provided with RNA messenger, they make the expected polypeptides. Later, just before the first differentiations of the cells begin to be determined, and thereafter, remarkable progressive changes appear in ribosomal activity. When extracted from the cells and offered exogenous messenger RNA, they form decreasing amounts of the corresponding polypeptide. The ribosomes appear to have become programmed with the organism's own gene-produced RNA messengers. In other words, before cell differentiation becomes determined, the genes are largely silent; later they become much more active. This indicates that genes of higher organisms do indeed exist in states of varying activity and that cell differentiation is correlated with changing genic activity. These indications are confirmed by other studies which, as will appear, deepen or extend our understanding.

Obviously, the analysis would be greatly facilitated and strengthened if it were

possible to look at a chromosome and see whether it or definite parts of it, i.e., some of its genes, were actually active or inactive. Surprisingly enough, it now looks as if this is indeed possible in favorable cases. In them, inactive genes or chromosome parts or whole chromosomes turn out to be highly condensed or tightly wound up. Genic activity is correlated with extension, with unwinding of the chromosomal thread. One striking and widely known recent example has grown out of the work of Barr and Mary Lyon.[4] It concerns the sex or X chromosome in female mammals. The female normally has two, the male one, in each cell. But in certain cells of the female, one of the two X chromosomes becomes tightly coiled up at a certain stage of development and remains so thereafter in all descendants of such cells. Which of the two coils up and which does not is a matter of chance; different ones do in different cell patches of the same female. Correspondingly, when the two X chromosomes of a female bear different allelic genes, only one is expressed in some cells, only the other one in other cells. Such females are for these traits a patchwork or mosaic of differently differentiated cells. The suppression of the action of one gene is correlated with the tightly coiled condensed state of one X chromosome. The expression of the other gene is correlated with the uncoiled extended condition of the other chromosome. The active and inactive states of the genes of a whole chromosome are thus visibly evident.

Equally striking and convincing visible evidences have lately been adduced for the localized activity of small regions of a chromosome, indeed for a single gene in the most fully analyzed case, while neighboring regions and their genes remain inactive. This work has been made possible by the existence of two kinds of extraordinarily large chromosomes (Fig. 1). One kind occurs in the oöcyte or pre-egg cells of amphibia. These so-called lampbrush chromosomes are characterized by paired lateral loops of various sizes and shapes alternating in definite linear sequence with intervening sequences of granules of varying sizes composing a dense axial strand. Gall and Callan[5] showed that RNA synthesis occurs on the loops, not on the dense axial strand. Their evidence indicates that the loops are formed by uncoiling of granules on the axial strand, and that axial granules are formed by tight coiling of the loops. The loops thus appear to be active genes, the granules inactive genes. Recently Izawa, Allfrey, and Mirsky,[6] following up earlier evidences that histones are associated with genic inactivity, showed that loops can be made to cease forming RNA and to regress to the condensed granular state by adding arginine-rich (but not lysine-rich) histone or by adding actinomycin D which blocks formation of RNA by DNA. Chemistry and the eye have again reinforced each other. Localized chromosomal sites, presumably individual genes or clusters of them, can exist in two reversible states related to the kind of polypeptide present at the site. The active RNA-forming state is uncoiled and spun out; the inactive state, during which RNA is not made, is condensed and associated with arginine-rich histone.

In most essential respects, comparable results have been obtained by Beermann[7] and others with the other kind of large chromosome found in the cells of certain tissues in the larvae of some insects such as the fruit fly and Chironomus. These chromosomes are really bundles of hundreds or thousands of identical stretched chromosomal threads arranged side by side in register. This association in effect magnifies the detailed structure of the chromosomes by two or three orders of mag-

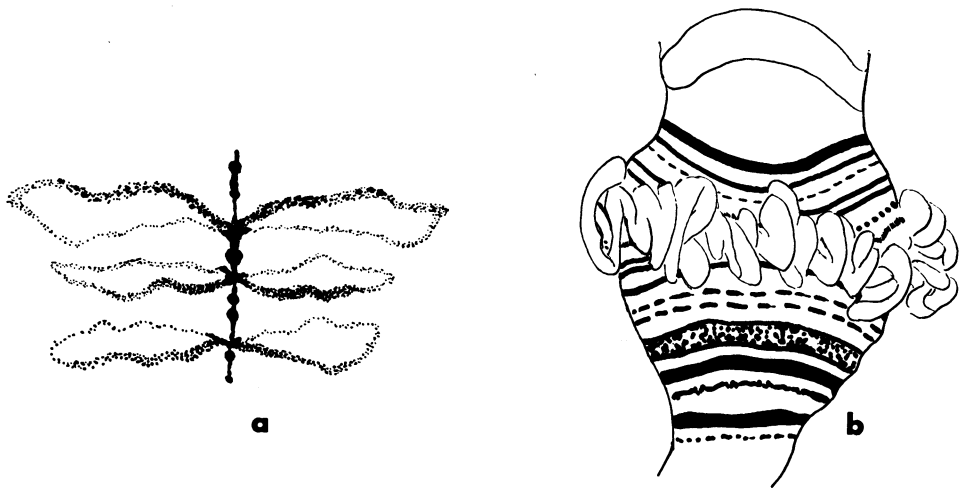

FIG. 1.—(*a*) Short section of a giant lampbrush chromosome from an amphibian oöcyte showing lateral loops extending from axial strand marked by sequence of granules. (Redrawn, after Gall.[5]) (*b*) Short section of a giant chromosome from cell of an insect showing linear sequence of bands with one of them opened up into a puff. (Redrawn, after Beermann.[7])

nitude and reveals a species specific normal linear sequence of bands, varying in width, form, and structure, and of thickenings of varying degree called Balbiani rings or puffs. Comparative studies revealed that a given position in a chromosome sometimes appeared as a puff and sometimes as a band. In fact, a puff is an opened-up band, opened up into what seems to be a looped thread. These are reversible changes. The puffs of these giant chromosomes are like the loops of lampbrush chromosomes; the bands are like the granules in the axial strand. The parallel is chemical as well as morphological. The puffs are sites of RNA synthesis and are free of arginine-rich histone; the bands possess arginine-rich histone and do not detectably synthesize RNA. In both kinds of chromosomes localized chemical activity and inactivity are rendered visible. But in these giant chromosomes the relations to the genes are much better known. Many genes of the fruit fly were long ago mapped on them, the positions of the genes being defined in relation to the visible chromosomal bands. In some cases a particular gene was closely correlated with a particular band or puff. So, in view of the chemical studies, we may regard the gene as inactive when its position in the chromosome is occupied by a band, as active when that position appears as a puff. These facts already show that a given gene is sometimes active, sometimes inactive.

Now it should be possible to ask and answer the question of whether differential genic activity is correlated with cell differentiation. And Beermann has just about done this. He found that only about 10 per cent of the bands were in the puffed, active form in any one cell at any one time, about 90 per cent being in the *in*active condition. Even more important, he observed that different bands or genes were puffed (and therefore active) in different kinds of cells and in different stages of development. Impressive as such evidence is, the most decisive evidence came from comprehensive study of one particular chromosomal spot. This spot was puffed only in certain salivary gland cells; in all others it appeared as a band. Moreover, the cells in which it was puffed, but no others, formed in abundance a

distinctive substance, a granular secretion. That the formation of this substance depended on a single gene located at the spot where this puff appeared in the secretory cells was shown by crosses to a nonsecreting strain and by cytogenetic studies which localized the gene at exactly that spot in the chromosome. In the nonsecreting strain, the spot is occupied by a band which fails to puff in the cells that correspond to the normal secretory cells but fail to secrete in this strain.[8] This elegant one-gene one-band analysis shows clearly that activity of the secretion-determining gene is correlated with its puffed appearance, while its inactivity is correlated with its appearance as a condensed band; and that the activity of this gene is correlated with the differentiation of a cell into a secretory cell.

Of course, we would now like very much to know what makes this gene become active and how it happens that it becomes active only in these particular cells and just at a definite stage of development. Very little is known about this sort of thing in any organism. But some promising starts have come recently from Beermann's laboratory. For example, Clever[9] has noted that a definite pattern of localized puffs appears in a definite time sequence following administration of the molting hormone, ecdysone. It is as if the hormone, directly or indirectly, activated or derepressed a few genes and as if the actions of these genes derepressed other genes, and so on in a definite sequential series. This at least suggests a plausible partial answer to our questions. The derepression of one gene may be dependent upon the prior derepression of another gene. Hence, whatever stimulus derepresses the secretor gene may be able to do so only in cells in which certain other particular genes are derepressed. In this way, although the immediate stimulus might be a circulating hormone to which all cells are exposed, only those cells possessing the appropriate pattern of other derepressed genes will respond by the derepression of the secretor gene. This of course pushes back the problem to accounting for the origin in the first place of different patterns of derepressed genes in different cells. One clue to this has come from other aspects of Clever's studies. He finds that mere variations in the amount of a hormone, or in the relative amounts of two hormones, reaching a cell can bring about different puff patterns. Hopes are thus high that such studies will step by step fill in the large remaining gaps in our understanding of how progressive changes in differential genic activity can bring about the precise sequence of cellular differentiations characteristic of normal development.

Quite another kind of study has also been tying differential genic activity to cell differentiation. Niu, Yamada, and others have been exposing cells of one type to the most direct products of genic action, RNA and protein, extracted from cells of another type. They report that these extracts induce the exposed cells to differentiate, to form products made specifically by the cells from which the extract was obtained.

Niu and collaborators[10] have explored the effects of extracted RNA using amphibia, mice, and chicks. They report that RNA extracted from cells of the thymus gland, from liver cells, and from kidney cells induces the appearance in other cells—even in tumor cells—of specific features of the cell from which the RNA was extracted. For example, liver RNA induced tumor cells to produce serum albumin, glucose-6-phosphate, and tryptophan pyrrolase, all specifically liver cell products. Kidney RNA did not induce production of these proteins, but did induce

production of a characteristic kidney protein, L-amino acid oxidase. Niu's analysis, based on experimental blockage (by actinomycin D) of synthesis of RNA by the genes of the recipient cell, indicates strongly that some of the introduced RNA functions directly in protein synthesis, at least for an hour.

Of even greater interest is the claim that the changed pattern of protein synthesis continued during growth as long as the cells were followed, for about 50 cell generations. This implies that the genes of the recipient cell were "turned on" to make the same proteins as those made by the introduced RNA. Taken at face value, this would imply that the putative RNA extract contained both the genic messenger and an inducer or derepressor to activate the corresponding gene; and, more important, that these differences between liver cells and kidney cells, for example, are due to the activity (and inactivity) of different genes in the two kinds of cells.

While Niu reported the active material in his extract to be RNA, not protein, Yamada[11] and others reported just the reverse. This puzzling discordance remains unaccounted for.

Up to this point, the examples cited have correlated cell differences with the activity of different genes as if genes existed in only two alternative states, active and inactive. Quite aside from the technically difficult problem of ascertaining whether inactivity is total or just so low a level of activity as to escape detection, there is excellent evidence that the same gene can be active to varying degrees and that such quantitative differences in genic activity are also important in cellular differentiation. A beautiful example of this is found in the work of Markert[12] and others on the enzyme lactate dehydrogenase.

This enzyme, like a number of others, exists in a group of somewhat different forms constituting what is called a set of isozymes. In the case of lactate dehydrogenase, there are five isozymes, and any cell that has one usually has all. But the cells of different tissues or organs or the same tissue or organ at different stages of development have different proportions of the five isozymes, sometimes very different. These differences have been shown to be due to differences in the relative activities of two genes. The two genes make two different polypeptides which are the building blocks of the enzyme. Each enzyme molecule is a tetramer, consisting of four polypeptides. The four may all be one kind of polypeptide or all the other kind or any one of the three possibilities of combining the two kinds of polypeptides in groups of four, i.e., 3:1, 2:2, or 1:3. The relative amounts of the five isozymes formed when the two kinds of polypeptides are mixed in varying proportions *in vitro* is exactly what would be expected by chance combinations. The same chance relative amounts are found in different kinds of cells, indicating that combinations are also random in the cells and depend upon the relative available amounts of the two kinds of component polypeptides. In other words, since the polypeptides are genic products, the different proportions of the isozymes found in different kinds of cells appear to be due to relative differences in activities of the two genes involved.

In sum, it is now abundantly clear that in higher organisms, as in microorganisms, genes may be turned off or on and turned on to varying degrees, and that such variations in genic activity result in differences among cells that have the same set of genes. The mechanisms controlling genic activity are just beginning to be explored experimentally with some success. On this phase, theory is far ahead of

knowledge, so that while the specific mechanisms are largely unknown, in principle it is easy to see how controlled variable genic activity could explain many well-known problems of cell differentiation. For example, one can now readily imagine why the cells that give rise to red blood cells differentiate so as to make hemoglobin, why they make different kinds of hemoglobin in different stages of development, why a liver cell differentiates to make glucose-6-phosphate while a kidney cell differentiates to make L-amino acid oxidase. Each of these differentiations doubtless depends upon specific substances which activate or derepress the genes that make the differentiating proteins, for it now appears that many genes remain inactive unless specifically derepressed. This makes sense of the fact that when differentiated cells are removed from the body and cultured in isolation they commonly cease to make their characteristic proteins: removal from the body has removed them from the source of their genic derepressors. The technical achievements of Eagle, Puck, and others[13] in culturing mammalian cells *in vitro* will thus have to be matched by discovery of the relevant derepressors before this great new cellular technique can be fully exploited in the analysis of cell heredity and differentiation. However, the fact that the future can be defined in these terms only serves to emphasize the importance already attributable to variable genic activity in cell differentiation.

Other Aspects of Cell Differentiation.—The simplest assumption to adopt concerning the basis of cell differentiation is that it is all due to one fundamental process. Since one process—variable genic activity and its regulation—is already known to be of wide applicability, this is clearly the candidate for the universal and exclusive basis of cell differentiation, if there is only one. To enable it to account not only for cell differences in the kinds and relative amounts of proteins—the most direct durable products of genic action—but also for cell differences in other substances and in the totality of cell structures and functions, one need only add the ancillary hypothesis of automatic self-assembly of the direct and indirect products of genic activity. According to this hypothesis, the gene-produced proteins interact by purely random collisions, as illustrated already in the formation of the tetramers of the lactic dehydrogenase isozymes. Just how far such automatic self-assembly can go in accounting for cell differentiation is of course not yet known. It is obviously good scientific procedure to refuse to multiply hypotheses until the facts demand it. Is there then any present compelling evidence that variable genic activity and self-assembly alone cannot account for some kinds of cell differences? If so, what other principle or principles are involved?

We may first of all eliminate certain superficial exceptions. For example, some cell differences are clearly due to the presence of viruses in the cells. But this is hardly a difference in principle, for these differentiations are doubtless traceable also to genes and their action, but the genes belong to the parasite or symbiont instead of the host cell. In like manner, evidence is now accumulating that genic material, DNA, occurs in cytoplasmic structures such as plastids and perhaps mitochondria. Regardless of whether such DNA represents independent cytoplasmic genes or whether it is derived from nuclear genes, again no new principle beyond variable genic activity and self-assembly is yet obviously required.

Difficulties might be expected in proceeding from random collisions of molecules to nonrandom organization. But even here the recent elegant and penetrating

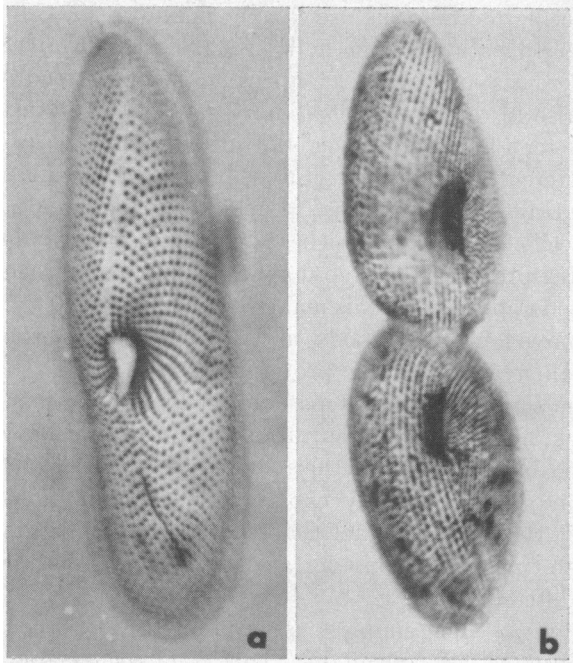

FIG. 2.—(*a*) Photo of pattern of rows of granules (silver impregnation method) marking the bases of the cilia in the unicellular animal *Paramecium aurelia*. The central comma-shaped space marks the position of the mouth and gullet; the long dark line in the lower part of the cell is the anus. (*b*) Same species, prepared in same way, showing a stage of transverse division into two daughter cells. Note the recreation of identical patterns and structures in both cells.

studies from the laboratories of Kellenberger[14] and Edgar[15] on the control of virus organization indicate that genic action somehow also directs an amazing degree of precise nonrandom structural patterning. Yet a virus is far from a cell. Such analyses seem more relevant to the determination of cell organelles than to the determination of total cellular organization. A virus does not grow and divide like a cell. Its nucleic acid replicates and its other structures are separately formed, the parts later coming together in the final organization. On the contrary the integrity of nonrandom cell structure persists throughout growth and division which immediately suggests that the pre-existing structure plays a decisive role that may not be explicable by mere random self-assembly of genic products.

Consider, for example, a highly organized asymmetric cell such as the Protozoan, Paramecium (Fig. 2). It divides transversely and yields two identically structured cells from one. This could not possibly be achieved by a mere transverse cut, for that divides it into two very different halves. The cell, however, achieves production of identical daughter cells by a complicated reproduction of all its precisely localized structures with distribution in such a way as to reconstitute the original pattern in both daughter cells.

Although mere observation suggests that more than mere self-assembly of genic products is involved, experimental analysis is obviously required. What is needed is a thoroughly analyzed test case in which cells that are identical in the genes present and in the genes which are active differ not in the kinds of proteins and other substances and structures which are present, but only in their arrangement. Such differences have long been experimentally created and studied in some of the large, complex, unicellular, ciliated Protozoa, especially in Stentor by Tartar[16] and in Blepharisma by Suzuki.[17] Comparable intracellular operations have recently been made on the egg of the amphibian, Xenopus, by Curtis.[18] All of this work points in the same direction, but it lacks the final critical step of genetic analysis to test whether differences in genes or genic action were excluded. In fact, that step has thus far been taken only in our work on Paramecium[19] (Fig. 3).

We experimentally altered this precisely regular normal pattern of structure in

various ways to yield, for example, cells with two or more cell mouths and gullets in various positions. You might suppose that such imposed upsets of normal cell structure would either be lethal or be rapidly corrected by the cell's genic actions, as the principle of self-assembly would lead one to expect. On the contrary, the bizarre cells are quite viable, the imposed differences persist, and they are as a rule inherited by the progeny at successive cell divisions and even through sexual reproduction. Moreover, what amounts to transplantation of nuclei in both directions between these and normal cells, as well as standard breeding analysis, showed that these hereditary cell differences are not due to differences in either the genes present or genic activity. They are due only to the differences in initial cell structure and organization.

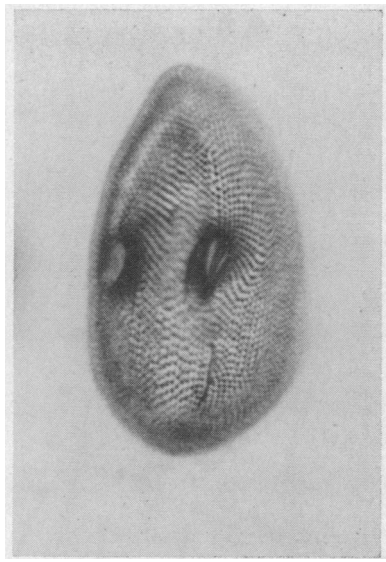

FIG. 3.—Photo of Paramecium with two mouths, two anuses, and two mid-ventral patterns of ciliary bases.

This is perhaps shown most simply and impressively by the simplest cell difference of all. Beisson[20] discovered how to create cells in which one or several of the 70-odd longitudinal rows of surface units was inverted, and we have followed the fate of inversions. As Figure 4 shows, each unit is very asymmetrical. For example, the fiber emerging from the base of a cilium normally is on the animal's right and extends forward. The units of an inverted row have their fibers emerging on the left and extending backwards. Such experimentally produced changes have been perpetuated during fissions for over a year, during which more than 700 cell generations have taken place. As the length of a row is doubled in each cell generation, the original row (about 125 μ long) has grown to $(2)^{700}$ or more than $(10)^{210}$ times its initial size. Had it been possible to keep all the progeny and place them end to end, the total length would now be roughly $(10)^{200}$ kilometers or about $(10)^{20}$ times the distance from the earth to the sun. This is perhaps enough to show the extreme stability and determinism of a merely structural intracellular rearrangement in the absence of differences in genes or gene action. It is a consequence of the fact that during growth new surface units appear within an existing row of units and are oriented in the same way as the units that are already there. This ordering and arranging of new cell structure under the influence of pre-existing cell structure I call "cytotaxis." Paul Weiss[21] has long adduced evidence for the same sort of thing, which he refers to as "macrocrystallinity."

This, I submit, is a second principle of cellular differentiation, one that is quite distinct from variable genic activity. The cell differences we have just been discussing are not characterized by different kinds of substances or structures, but by different numbers or arrangements of structures. Their perpetuation shows that they can be decisive in cell differentiation. Self-assembly of genic products alone cannot account for this. The place and orientation of the assembly of genic products is also determined by pre-existing assemblies of molecules and structures.

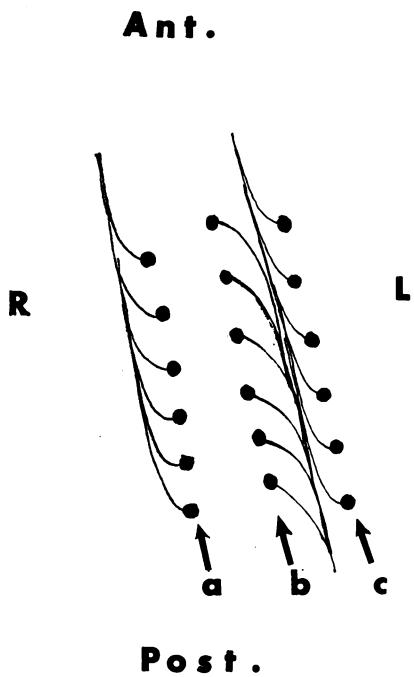

FIG. 4.—Diagram of small piece of the surface of Paramecium showing six surface units in each of three rows (*a*, *b*, *c*). In rows *a* and *c*, the fibers emerging from the ciliary base come off on the cell's right (*R*) and extend anteriorly (*Ant.*); in row *b*, the fibers come off on the left (*L*) and extend posteriorly (*Post.*). Normally, all rows are like *a* and *c*; but the abnormal orientation of a row (*b*), when present, is inherited.

The picture that emerges is no less deterministic or molecular than self-assembly, but it is fuller and truer: there is more than "self" to the mechanism of assembly; it includes pre-existing and independently modifiable assembly.

Thus, variable genic activity is decisive in cell differentiation by determining directly the kinds and proportions of molecular species present; but pre-existing cellular structure is also decisive cytotactically by determining the location and orientation of these molecules and others formed from their reactions. As Weiss[21] and Grobstein[22] have argued and as embryologists have long believed, the further one goes away from direct genic action in the economy of the cell and organism, the more important other factors become. While the genes determine the molecular building blocks and, through their properties, the kinds of molecular associations that can occur, the associations that actually do occur depend also on those that already exist. Calling these cytotactic events epigenetic or epigenic should not obscure their degree of independence or their decisiveness for the end result of cellular differentiation.

Prospects.—Where then are we heading in the further analysis of cellular differentiation?

The main present gap in knowledge about variable genic action is in the molecular species and events directly and indirectly involved in genic derepressions. It is especially important to discover the mechanisms of specificity by which one and not another gene is activated or repressed. After these things are known, it should become possible to understand the regular progressive *series* of genic activations and repressions which lead to normal developmental cellular differentiations. I have said nothing about interlocking pathways of metabolism with their feedback inhibitions, compensatory regulations, and stimulations, though they are obviously important in cellular function and differentiation. Much is already known about them. Much more needs to be discovered.

There are already strong indications that certain aspects of genic action in higher organisms are different from those in bacteria. This is not surprising in view of their more highly evolved chromosomes and nuclei. For example, genic messenger RNA is exceedingly short-lived in bacteria, of the order of minutes. The already mentioned results of Niu and those of others indicate longer life of the RNA messengers in vertebrates, of the order of an hour at least. The remarkable development

of differentiations in the giant unicellular Alga Acetabularia,[23] after removal of the nucleus, suggests that its messenger RNA may be stable for weeks.

Gibson and Beale's[24] remarkable results on an RNA intermediate between a gene and a trait in Paramecium, presumably a messenger RNA, seem to show that it is infectious (like the RNA in Niu's work), and immortal. A mathematical analysis by Reeve and Ross[25] indicates that this RNA may have a weak capacity to multiply. Recently, Gibson and I[26] have introduced this RNA into another, not even closely related, cell (Didinium). It then multiplies fast and without limit in the absence of the gene of Paramecium that apparently produced it.

Does this mean, in spite of the recent evidence for specific polymerases essential for the replication of each RNA virus, that messenger RNA's may also, under certain conditions, be capable of long persistence and even replicative reproduction? If so, this could be the basis of long persistent or permanent cellular differentiations in animals. On the other hand, there are also mechanisms in bacteria for rapid cellular destruction of messenger RNA and, in Paramecium, for nearly complete inhibition of multiplication of the one known to be capable of rapid multiplication in a foreign cell. Such inhibition would help to keep cells from becoming cluttered with the apparatus of previous differentiations. Higher plants have amazing capacities to develop whole plants from body cells or even from tumor cells, as Steward[27] and Armin Braun[28] have shown. Clearly, organisms differ in the stabilities of their differentiations. These may be traceable, at least in part, to differences in the stabilities of their RNA messengers and/or their genic activations and repressions.

Perhaps the most important prospect for future theoretical work is to profit by the way in which success has been achieved in viral and bacterial work and to recognize that systems in microorganisms, in spite of their possible primitiveness, have much to tell about what goes on in higher organisms. The Monod-Jacob attack on the β-galactosidase locus in the colon bacterium, pursued for many years, gave us the concept of regulation of gene activity on which the major current attacks on cellular differentiation are based. Indeed, the successes and consequent possibility of applications, of microbial work stem from concentrated attacks on relatively simple systems, often single genes. This should be a model for other work on cellular differentiation.

The slime mold mentioned earlier is such a simple system and concentrated work on it by K. Raper, John Bonner, Maurice Sussman, Barbara Wright, and others,[29] using genetic, chemical, and biological approaches has yielded very fruitful results. Recently, some[30] have recognized the promise of analyzing the amazing synchronized and controllable differentiation of an amoeba into a flagellate, in Protozoa such as Naegleria.

In view of the central problem of genic control systems, I think it far better to select a simple cellular differentiation system in a cell that can be bred and subjected to standard breeding analysis. That is why we have used Paramecium. Recently I[31] have found sexuality in, and have begun to exploit, a simpler system with the same advantages, a suctorian, Tokophrya (Fig. 5). This cell, under controllable conditions, transforms in 3 min from a free-swimming ciliate into an immobile, attached, stalked tentacled cell. When the immobile complementary mating types are confronted at a distance, each induces the other, apparently via

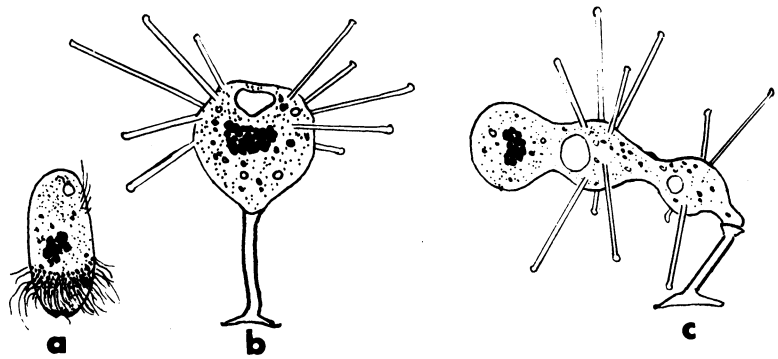

FIG. 5.—Diagram of three stages in life of the unicellular animal, Tokophrya. (*a*) Free-swimming, ciliated "young." (*b*) Nonsexual phase of attached adult, lacking cilia, but bearing tentacles and stalk. (*c*) Sexual phase, with amoeboid features.

diffusible substances, to assume amoeboid characteristics. I have already a gene mutation affecting one differentiation, and we—my student Laura Colgin and I—are discovering how to control other differentiations. This material seems extraordinarily favorable for deep penetration into problems of cellular differentiation.

With respect to future possible practical applications, knowledge of how to turn genes on and off and how to affect other cellular differentiations promises tremendous medical uses, as Tatum[32] long ago foresaw. It is not too much to imagine that tumor cells as well as other pathological forms of cells, perhaps even some aging changes, may prove reversible if we learn how to regulate the activities of the relevant genes. Niu[10] appears to have gone a long way toward this already. This basic level is obviously the one to attack, not the fully developed end results as nonscientists imagine, if we are to ameliorate the physical, and hence the total, well-being of the human individual. On both the theoretical and the applied sides, the analysis of cell differentiation—already brilliantly begun—indeed holds prospects full of promise for science and for Man.

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[8] The failure to puff indicates that this gene makes, not a "wrong" product, but no product.

In the hybrid the gene puffs in one chromosome of the pair, but remains a condensed band in the other. Hence, failure to puff in the hybrid can hardly be due to absence of the proper stimulus; it is seemingly due to some defect in the response mechanism, presumably in the "operator" on the "operon" hypothesis.

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