## SUNDER LAL HORA MEMORIAL LECTURE, 1965

# SOME ASPECTS OF DIFFERENTIATION IN PROTOZOA

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(*Delivered on October 7, 1966*)

I would like to express my thanks to the National Institute of Sciences of India for the honour they have done me by choosing me for the award of the Medal named after one of our distinguished Biologists, the late Dr. Sunder Lal Hora.

The enormous interest in the phenomenon of differentiation in recent years as a means of understanding diversity in nature has been extended to unicellular organisms, where differentiation is an expression of changes taking place within the confines of a single cell.

All diversity at cellular, tissue and organismal level is due to the process of differentiation. The development of a single cell, the zygote, into a complex individual animal involves a variety of changes and ultimately results in a harmoniously functioning organism adapted to its environment. During the development of higher animals, the fertilized egg divides repeatedly and the daughter cells undergo a complex series of movements to acquire new spatial relationships. Interaction among the cells leads to histological differentiation. The tissues are then shaped into organs which are the components of an integrated organism.

In some animals such as *Ascaris*, the products of nuclear division during early cleavage show visible differences. However, differentiation at such an early stage of development is rare and in the majority of animals, the nuclei formed during early cleavage do not show differences. In amphibian embryos, it has been possible to shift the plane of third cleavage from horizontal to vertical by application of pressure. In spite of the resulting abnormal distribution of nuclei in the various cytoplasmic regions, these embryos develop normally. This experiment indicates that nuclei derived from early cleavages have identical developmental potencies and the cause of differentiation should, therefore, lie outside the nucleus, perhaps in the cytoplasm.

Whether the nucleus during development undergoes any change in its ability to support the complete development of an organism was studied by Briggs and King (1959) in their celebrated experiments of nuclear transplantation. They introduced nuclei from various differentiated cells of blastula, gastrula and neurula stages into enucleated eggs of the frog and studied their

development. Extensive studies by these authors have indicated that the nuclei do undergo some sort of differentiation and that they gradually lose their ability to support complete normal development in enucleated eggs.

Differentiation of cells into distinct types is due to the synthesis of specific proteins. The genetic control of this process has been revealed recently. In embryos, visible differentiation is preceded by synthesis of RNA which is probably the same as 'determination', a term generally used in experimental embryology. In early cleavage, there is very little synthesis of proteins. The process of cleavage is unaffected by any treatment which later interferes with protein synthesis. Thus, radiation (Neyfakh 1964), 'pollution' of nucleus by replication in a 'foreign' cytoplasm (Moore 1962), permanent and replicable changes in the nuclear material due to proteins injected into the zygote (Markert and Ursprung 1963) or interference with RNA or protein synthesis by various means (Brachet 1960) have little influence on cleavage but have considerable effect on gastrulation and later development, which depend on synthesis of proteins. Some proteins necessary for cleavage are known to be synthesized immediately following activation in echinoderm eggs. This process is, however, resistant to actinomycin D and is now known to be due to the presence of stable messenger RNA produced before fertilization (Gross 1964).

Since the basis of cell differentiation is synthesis of proteins, attempts have been made to formulate a general theory of differentiation which would include induction of enzyme synthesis in microorganisms as well as in cultured mammalian cells. However, at present, there is no experimental evidence indicating that such a general theory is possible (Wilt 1964). In the differentiation of animal embryos, the mechanism regulating gene activity is more complicated than in microorganisms. Gene activity in developing embryos has a spatial as well as a temporal pattern. In other words, identical genomes function differently in different parts of the embryo and also at different periods. The basis of spatial differences in gene activity may well be due to differences in the cytoplasmic environment of the genome. The temporal pattern is, however, more difficult to understand. Genes responsible for synthesis of very specific proteins like haemoglobin remain dormant till the erythroblasts are formed. The haemoglobins of the human foetus and adult are known to be different. What regulates the time pattern in the activity of genes responsible for synthesis of the different haemoglobins is not known. The multiple molecular forms of the enzyme lactate dehydrogenase show not only specific proportions of the 'isozymes' in different types of cells but also a changing pattern in development (Markert 1963). How gene activity is regulated so as to bring about this complexity is not known.

While differentiation in higher animals is interesting enough, that in unicellular animals like the Protozoa is vastly more so. This is due to the fact that it takes place within the confines of a single cell and, whatever diversity

prevails among the different species and genera, must find expression in the structure and behaviour of parts of a single cell. The Protozoa exhibit such a wide range of variation, in organization as well as function, in size, habitat and ecology, that they constitute a whole microcosm. Compare, for instance, the simple naked protoplasmic body of *Amoeba* with the amazing complexity, symmetry, beauty and architecture of a Radiolarian, with which it is not too distantly related, with the complex intracellular structures in parasitic flagellates and with the intricate mechanisms of reproduction in ciliates. That all these are unicellular organisms included among the Protozoa holds us in constant wonder.

It is evidently impossible for me to review the whole field of differentiation among the Protozoa: it would be a stupendous task. Perhaps I might be permitted to refer to some aspects of differentiation with which we have some personal and close acquaintance. It is also my endeavour to delineate the several levels at which differentiation takes place in these organisms.

As a first example, I would like to choose the Foraminifera. Foraminifera are mostly marine Rhizopods secreting a shell of calcium carbonate in which the protoplasm is confined and through the pores of which it comes out as greatly elongate, streaming, anastomosing pseudopodia. The shell consists of either one chamber or, more usually, of several chambers, communicating with one another in a rather complicated fashion. In several genera, like *Rotaliella*, there is an alternation of generations, the asexual agamont and the sexually reproducing gamont succeeding each other in a regular sequential relationship. Grell (1964) found that in the agamont, after fertilization, the zygote nucleus divides twice to give rise to four nuclei, of which one differentiates into a large, vegetative nucleus, and the others become the generative nuclei. Meiosis occurs in the small nuclei; only they are destined to give rise to the gametes. The large vegetative nucleus, on the other hand, enlarges, develops a nucleolus, exhibits great metabolic activity and is responsible for the growth of the animal. Once this growth is achieved, it degenerates and dies. One cannot fail to see the parallel between this situation and the one that obtains in the relationship of the macro- and micronuclei in ciliates.

Coming back to the differentiation of the vegetative nucleus in *Rotaliella*, why does only one nucleus (of the four) differentiate into a somatic nucleus? One may assume, on the analogy of other organisms and situations, that a certain determinative substance is present in the cytoplasm to which the one nucleus that is most susceptible is subjected and as a result of which differentiation occurs. However, this has not been proved. In fact, it would be difficult to prove it. What is proved, however, is the indispensability and inevitability of this differentiation. If the differentiating somatic nucleus is inactivated and rendered ineffectual by an ultra-violet microbeam, one of the three generative nuclei moves forward and takes its place. It develops a

nucleolus, enlarges and becomes a somatic nucleus. This can happen more than once, and as long as there is a stock of generative nuclei available, the differentiation of one of them into a somatic nucleus continues to occur.

A similar but perhaps more complex situation obtains in Myxosporidia, one of the sporozoan orders of the Protozoa. These are important parasites of fishes and are responsible for great fish mortality in several countries. The essential facts that concern us are these:

The life history begins with a uninucleate bit of protoplasm which, after repeated mitoses, takes on a plasmodial and syncytial character, resulting, in course of time, in a number of nuclei in the same protoplasmic mass. Some of these nuclei are reproductive and some are vegetative. The latter soon disintegrate. Each reproductive nucleus builds around it endogenously a small independent unit of protoplasm and becomes the fore-runner of all the complex organization of a spore. Its nucleus divides by mitosis into a large and a small nucleus, each in turn divides again, producing four nuclei, two of which are large and two small. The two small ones form the wall of a structure called the Pansporoblast; they later die. The large nuclei divide by mitosis producing six daughter nuclei. The fates of these six nuclei are diverse; two form the spore valves; two form polar capsules, highly characteristic structures of this order. The other two are the definitive nuclei of the sporeplasm which along constitutes the effective infective body.

The diverse destinies of mitotic products of a single original nucleus disposed within the same cytoplasmic *milieu* are extremely interesting. Here, the products of mitosis not only differentiate into generative and somatic nuclei as in the Foraminifera but they initiate the development of a great variety of structures.

Protozoa offer many examples where nuclei derived by mitosis become differentiated from one another even though they are located in a common cytoplasm, and display different fates. But perhaps in no other group are they as extensive and as instructive as in the ciliates. The origin and differentiation of the two nuclei in the ciliates has been a subject of special interest to us over the years. In the ciliate protozoa, the nuclear apparatus is divided into a large macronucleus and one or more small micronuclei. The large majority of ciliates display this nuclear dualism which has an underlying basis, not only of a difference in size but also of structure, mode of division and function. The macronucleus is several times as large as the micronucleus (sometimes as in Spirostomum, several thousand times as large), divides by an apparent amitosis and has a 'vegetative' function. The micronucleus is small, divides by mitosis and is regarded as a generative nucleus. The interest lies in the fact that both these nuclei, so diverse in appearance, structure and function, are the differentiated products of mitotic division of an original single nucleus.

Conjugation is a highly characteristic method of sexual reproduction in ciliates. It involves the temporary union of two animals belonging to two different mating types. In *Paramecium aurelia*, during conjugation, the macronucleus disintegrates, the two micronuclei divide twice producing eight nuclei. Of these, only one is chosen for a further division and this results in two nuclei in each conjugating animal. There is a reciprocal fusion of nuclei in the two animals resulting in the production of a synkaryon or fusion nucleus. The question is, of the eight products of micronuclear divisions, only one is chosen for a further division; which is this and what is the basis on which this is done? While in examples like *Paramecium aurelia* with two micronuclei in the vegetative animal, the products of the first two divisions number eight, out of which only one is chosen for the third pregamic division, in cases where the original micronuclear number is very large as in *Spirostomum* (which has a hundred or more micronuclei), the situation becomes very highly interesting and the method of selection out of the many hundreds of products of micronuclear divisions seems unaccountable. In *Paramecium*, Sonneborn (1947) found that the one nucleus which finds itself in or near the paroral cone becomes successful in going through the third division, while all the rest degenerate. He felt that the cone region had a substance which protects the successful nucleus from some inhibitory substance present in the cytoplasm, which destroys the unsuccessful nuclei. He also found a mutant in which no micronuclear division product reaches the cone, resulting in the degeneration of all of them, thus making conjugation ineffective. The occurrence and continued operation of two different and antagonistic substances, one inimical and the other protective, within microns of each other in the same cell cytoplasm, is remarkable and offers a unique challenge to the investigator; but I must say neither the biologist nor the biochemist has been able to make attempts to solve it.

It is also possible that the nucleus itself has some positive role in this matter. Kimball (1964) and his associates feel that some kind of selection among the nuclei operates, resulting in the final choice of one. In *Spirostomum*, as I have just said, several hundred micronuclear products are present, out of which one is chosen for the third division. In this animal, we have found for the first time evidence for the presence of a specialized area in the cytoplasm in which the successful micronuclear product finds itself (Seshachar 1965*b*). At the end of the second pregamic division in this organism, in each exconjugant there appears beside the fusion line a clearly marked intensely basophilic region with a high concentration of RNA, in the centre of which one single nucleus becomes placed and it is this that passes through the last pregamic division. It is not yet clear where this RNA comes from, how it becomes concentrated in this region and what role it plays in affording protection to the dividing nucleus. It appears clear, however, that the RNA

deposition in this specialized area takes place long before the second pregamic division is completed so that it seems likely that the nucleus itself has nothing to do with its deposition and concentration. At the moment, I shall be content to postulate that a nucleus which happens to find itself in this region of high concentration of RNA is the one that is afforded adequate protection for completing the third pregamic division, and that produces the pronuclei.

After every conjugation or autogamy, there is a complete reorganization of the nuclear apparatus. The most important events of this reorganization are the dissolution and disappearance of the macronucleus and the reconstitution of a new macronucleus as well as a micronucleus from the fusion product of gamete nuclei, called the synkaryon. So, at the end of every conjugation, each animal has a single nucleus from which is built up the diverse and complicated dual nuclear system. In the simplest cases, this synkaryon divides once giving rise to two nuclei, one of which enlarges and grows into the macronucleus, and the other becomes the micronucleus, a condition found in *Chilodonella* (Seshachar 1950*a*). In *Paramecium*, *Tetrahymena* and a few others, there are two divisions, resulting in four nuclei, two of which become macronuclei and two micronuclei. A few genera display greater complexity; there are three divisions resulting in eight nuclei, of which two become the micronuclei and six differentiate into macronuclei; this is the situation exhibited by *Vorticella convallaria* we studied some years ago (Seshachar and Dass 1951). The cause for this differentiation of one or more products of the mitotic division of a single original nucleus into macro- and micronuclei, as also the determinants of this number, which is species specific, are interesting. In *Tetrahymena*, the position in the cell is considered to determine this, the two nuclei placed anteriorly becoming the macronuclei and those placed posteriorly becoming the micronuclei (Nanney 1953). Whatever the validity of this concept, where the cytoplasm of the two regions of the cell appears to determine the direction of differentiation, it does not appear to govern in case of a number of examples where the two nuclei lie close to each other in the cell and still develop in two different directions. Both *Chilodonella* and *Spirostomum* offer such examples. In both, the first division products of the synkaryon are juxtaposed to or within microns of each other and still exhibit diverse fates. It would appear the nuclei themselves play a positive role in this differentiation.

It has been clear for some time that the macronucleus is concerned with metabolic activities of the cell while the micronucleus becomes specially active during conjugation, which is the important process of sexual reproduction in these organisms. That the macronucleus is indispensable to the animal has been shown by the fact that amacronucleate animals do not survive for any appreciable period. That the micronucleus is not quite necessary for

vegetative existence has been shown by the fact that amicronucleate strains of several ciliates have been maintained over long periods of time.

Another interesting feature lies in the fact that in the macronucleus of several ciliates, during vegetative divisions, the chromosomes do not become visible and the division itself has been regarded as amitotic. The macronucleus just splits into two equal or approximately equal parts. On the other hand, the micronucleus divides by mitosis and it has been possible in many cases to see the chromosomes.

However, in a number of species, chromosomes or chromosomal filaments have been seen in the *early* stages of macronuclear differentiation, sometimes very curiously organized, as in the case of *Stylonychia* (Ammermann 1965) where banded chromosomes of the kind noticed in the salivary chromosomes of Diptera have been reported by several workers. In all cases, quite soon after the early stages of differentiation, the chromosomes become obscured and at no time later are they visible as detectable structures. The nature of the changes taking place in the chromosomes in the vegetative macronucleus is entirely obscure and neither light nor electron microscopy has shed light on this. It is a matter of interest, however, that the macronucleus has large amounts of DNA. We were among the first to make DNA measurements in the ciliate nuclear apparatus 18 years ago (Seshachar 1950*a*, *b*; 1951; Seshachar and Dass 1954*a*, *b*) and since then, several workers have determined the DNA content of the macronucleus and the micronucleus in order to establish a relationship between the two.

It will be remembered that it was about this time in 1948-49 that the concept of constancy of DNA in tissue cells was developed and it was possible to relate the DNA content with the nature of ploidy in nuclei (Boivin *et al.* 1948). For instance, it was established that the sperm of an animal had half the amount of DNA that the tissue nuclei had. It was also possible to say that while in young animals, tissue nuclei, like those of the liver, are generally diploid, with age, higher degrees of ploidy obtain, the multiplication of DNA taking place mainly by endomitosis. By cytophotometric measurements, it has now been possible to estimate the amount of DNA in the macronuclei of several ciliates and compare it with that in the micronuclei. These measurements have great validity and it is possible to say that the macronucleus of many ciliates is a highly compound or polyploid nucleus, having arisen from a micronuclear product, where the DNA has repeatedly multiplied. Thus in *Paramecium aurelia*, the macronucleus has about 430 times as much DNA as the micronucleus, indicating a ploidy of 860 (Woodard *et al.* 1961). In *Bursaria truncatella*, Ruthmann and Heckmann (1961) have found a 5,000 ploidy in the macronucleus. Perhaps the largest amount reported so far has been from *Spirostomum ambiguum* where the macronucleus

has a DNA content up to 11,400 times that of the micronucleus, establishing a 22,800 ploidy (Ovchinnikova *et al.* 1965).

That still leaves the question of the arrangement and distribution of this DNA inside the macronucleus and the organization of chromosomes in it. As mentioned earlier, the large size of the macronucleus leads one to believe that it is polyploid, as also the large amounts of its DNA. However, whether entire sets of chromosomes exist separately in the macronucleus or the many thousands of chromosomes are haphazardly distributed within it remains a problem. Experiments have shown that both during reorganization after conjugation as well as during regeneration, a small portion of the macronucleus is sufficient to bring about complete reorganization and regeneration, which would mean that such a fragment would possess at least one representative of each chromosome characteristic of the organism. The concept of 'subnuclei' which was put forward long ago by Sonneborn (1947) has been examined both by light as well as by electron microscopy and in *Paramecium,* the latest efforts by Jurand *et al.* (1962, 1964) have proved ineffectual in demonstrating discrete units inside the macronucleus which could be designated as 'subnuclei'. Actually, several species of ciliates have been examined under the electron microscope and none of them has displayed a compartmentalization of its macronucleus into discernible units which could be called subnuclei. The macronucleus has remained structurally an intriguing organelle of ciliate morphology.

Recently, however, methods other than, and in addition to, electron microscopy have been applied to a few genera of ciliates, and these have yielded significant information in regard to the organization of chromosomes inside the macronucleus. In 1958, it was observed (Seshachar 1958) that the macronucleus of species of two genera, *Blepharisma* and *Spirostomum*, could be stretched mechanically to great lengths and the process yielded a number of greatly elongated DNA filaments. More refined methods, centrifugation and application of despiralizing agents, like KCN and NaCN, have yielded from the macronucleus large quantities of DNA filaments (Seshachar 1960). From these studies as also from electron microscope observations on these two ciliates, it is now concluded that the chromosomes of these two genera, and perhaps of several others, are in the nature of greatly elongated filaments disposed inside the macronuclear membrane in all directions and that by relatively simple methods it is possible to unravel these filaments from the confines of the membrane (Seshachar 1964, 1965*a*).

The great length of these chromosomal filaments as well as the lack of any visible organization in them would lead us to believe that the chromosomes, at least in these ciliates, are more or less uniform DNA-containing structures of great length. It has, however, been difficult to derive the long filaments of the macronucleus from the small chromosomal bodies seen in

micronuclear mitosis. The acceptance of the view forwarded by Grell (1964) that macronuclear chromosomes are compound structures obtained from an end-to-end alignment of many micronuclear chromosomes offers one explanation. Seeing that the macronuclear DNA is largely concerned with synthetic activity, this view is not forbidden. Actually, the idea of chromosomes consisting of a few but greatly elongated filaments is put forward by several workers, the latest of whom is DuPraw (1966) who has found greatly elongated filaments in the chromosomes of several organisms including man. It has not yet been possible to relate together, in the history of the development and differentiation of the macronucleus, the several events of chromosomes, their end-to-end alignment and the multiplication of nucleoli not to speak of the processes involved in the increase of nuclear membrane surface attendant upon the enlargement in size and increase in the number of chromosomes.

Perhaps the most interesting example of cell differentiation operating at an intranuclear level is illustrated by the differentiation of mating types in ciliates. A general outline of mating types in these organisms may be appropriate here. In many ciliates, conjugation occurs between animals of different origin and belonging to two different mating types, there being little or no conjugation between animals belonging to the same mating type. Metz (1954) has shown that the interaction between mating types in *Paramecium* is due to the presence of complementary substances, in the nature of proteins, on the surface of the animals. In certain strains of *Paramecium aurelia*, the inheritance of mating types is governed by the macronucleus (*see* Beale 1954). It will be recalled that in this organism, after conjugation, the synkaryon divides twice giving rise to four nuclei. Two of these four become macronuclei and the other two, micronuclei. The interesting thing is that these two macronuclei, derived by division from a single original synkaryon, are of two different kinds, representing two separate and complementary mating types. The two animals produced by the first fission of the exconjugant differ between themselves. It would, therefore, appear that during the process of macronuclear differentiation, a permanent or nearly permanent change occurs in it on the basis of which the mating type becomes fixed. Experiments have shown that mating type determination can be altered during a very short period of this differentiation, and once this is done, no further change is possible under any circumstances.

The point of interest to us is that here again, of the two products of mitotic division of the synkaryon, destined to give rise to macronuclei, one is different from the other. We know nothing of the basis of these differences and at the moment appear to be unable to account for them.

An even more complicated form of mating type inheritance has been found in some strains of another ciliate, *Tetrahymena pyriformis* (Nanney 1964). In this case, there are seven mating types instead of the two found in

*Paramecium aurelia*, and all of them are determined by the macronucleus. Each single nucleus, therefore, has the potentialities of developing, not only one or two mating types, but several, up to seven, in this case. These express themselves throughout the cell cycle and during fissions of the animal mating types are segregated and liberated. It would, therefore, appear that when the macronuclear anlage develops and its chromosomes multiply by some kind of endomitosis, not only is there a multiplication of its chromosomes but there is also a differentiation of some of them. If we accept the subnuclear constitution of the macronucleus, for which there is abundant genetic, but hardly any morphological, evidence, it would mean that to begin with, the macronucleus is simple but during its growth and development, it proceeds not only to multiply its subnuclei but also to introduce diversity among them during the process. These subnuclei are segregated at succeeding divisions of the macronucleus until finally each macronucleus contains identical subnuclei. Such a macronucleus would represent a single pure mating type.

However, as already pointed out, no morphological, visible evidence is forthcoming for the occurrence of subnuclei in either *Paramecium* or *Tetrahymena*, or for that matter in any ciliate that has been examined so far. That within a single macronucleus could be contained more than one representative element, liable to later segregation, is an example of differentiation which, to my mind, has no parallel. More recently, characteristics other than mating types have been investigated in *Tetrahymena pyriformis* and it has been shown that here also a diversity might occur within a single macronucleus. Certain immobilization antigens of *Tetrahymena pyriformis* show essentially the same pattern of inheritance as the mating types. A nucleus contains several antigenic specificities during the early stage of its development but as fission goes on, they become segregated and give rise to pure types. A very similar system has also been found for certain enzymes, the esterases and acid phosphatases in *Tetrahymena*. In early stages of clonal history, more than one, often several types are represented in the macronucleus but, eventually, they become separated off into single pure types (Allen 1961; Allen *et al.* 1963).

The subject of differentiation is one of vast interest and importance in Biology. It underlies the whole diversity and variegation in nature. In this context, I feel I need make no apology for the choice of this subject and I wish to close with the hope that I have been able to outline some, but by no means all, the problems that are seen in just one group of animals, the Protozoa.

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