Developmental Cytology . . .

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Developmental Cytology

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Foreword

. . . jedenfalls müssen wir nach unseren Resultaten von einer physiologischen Einheit der der Zelle zukommenden Chromosomen und also von einer im "Kern" repräsentierten physiologischen Einheit reden.

THEODOR BOVERI

The Sixteenth Symposium of the Society for the Study of Development and Growth was organized around the cell itself. Advances in biochemistry, immunochemistry, electron microscopy, as well as in cytogenetics and cytochemistry, are rapidly altering our view of cellular activity, as much by producing new solidly based factual information as by changing the framework of permissible speculation regarding matters beyond our present means of observation. From the many workers in these fields, it was possible to assemble a series of speakers to discuss authoritatively new work on chromosomes, nucleoli, cytoplasmic organelles, cellular chemistry, and immune properties in relation to development or at least to cellular differentiation. All this work involves techniques almost unknown a few years ago. Since the Second World War these techniques have been exploited, refined, and to an extent tamed. The present volume containing the written versions of the Symposium papers constitutes a report on the status of those aspects of cytology particularly interesting to students of development.

The Symposium was held at Kingston, Rhode Island, June 19–21, 1957. The Society is greatly indebted to the University of Rhode Island, to the local committee and all others who entertained its meeting so hospitably; to the National Science Foundation for a grant supporting the Symposium, and to numerous colleagues who have helped with editorial and other tasks.

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Yale University
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Nuclear and Cytoplasmic Differentiation in the Protozoa

JOHN R. PREER, JR.[1]

The protozoan geneticist finds useful the concept of the clone: the asexually produced descendants of a single protozoan. Although clones generally show great constancy, variations often arise. The problem of the origin and maintenance of such intraclonal variations is a long-standing one and has been the subject of many investigations. Since such studies encompass most of the questions raised by my topic, I shall limit my discussion to a consideration of some of the problems of intraclonal variation. It is significant (as many have pointed out) that the cells of a multicellular organism also constitute a clone and that the problem of cellular differentiation in multicellular organisms is therefore similar to the problem of variation within the clone in unicellular organisms.

Many variations are not inherited, being associated either with the different stages of the mitotic and meiotic cycles or arising from the action of differing environmental conditions. The changes induced by environmental differences, however, generally quickly disappear after a few generations when the variants are cultured under one set of conditions. This fact would indicate that the generation time is large in comparison to the time required for most biochemical reactions to reach their steady-state values so that new equilibria are rapidly attained. Furthermore, the loss or acquisition of substances accumulated or depleted as a result of differential environmental action should be rapid, for dividing cells undergo a complete molecular turnover in less than 60 generations. Thus the original molecules present in a cell used to start a clone will be progres-

[1] Zoological Laboratory, University of Pennsylvania. This work has been aided by grants from Phi Beta Psi Sorority and from the National Institutes of Health, Public Health Service.

sively diluted by cell division. After one fission the average number of the original molecules per cell will be reduced to ½ (or 2^{-1}) times the starting number; after two fissions, to ¼ (or 2^{-2}), and after 60 fissions to 2^{-60} times the starting number. Since it can be simply shown [2] that the starting number is surely less than 2^{60}, the average number of original molecules per cell after 60 generations is less than one, and the probability of a given cell having an appreciable number of those molecules is consequently very low.

Many variations, however, persist for many cell generations under uniform environmental conditions, i.e., they are inherited. Other variants are not completely stable but undergo slow changes with time. The question can be argued as to whether variants of intermediate stability (the *Dauermodifikationen* of Jollos or many of the changes associated with the meiotic cycle to be considered presently) should be classified as inherited or not. The label we attach to such phenomena, however, is clearly unimportant, while an understanding of the mechanisms of variation, whether of great or of intermediate stability, may be of considerable interest. We will consider a few of the major examples of stable and long-lasting changes.

Gene Mutations, Chromosomal Aberrations, and Changes in Ploidy

The best-known causes of stable variations in all organisms are gene mutations, chromosomal aberrations, and changes in ploidy.

Gene mutations in the protozoa, as in other organisms, are generally rare, haphazard, and nonadaptive. Thus they are not commonly encountered in clones of moderate numbers of cells. It might also be noted that only infrequently are gene mutations invoked as an explanation of cellular differentiation in multicellular organisms (see, however, Monod, 1947).

Chromosomal aberrations and aneuploid changes have been reported to occur with high frequency in the micronucleus of aging lines of *Paramecium* by Sonneborn and Schneller (1955a) and Dippell (1955). Such changes may also account for much of the nonviability occasionally encountered in clones of paramecia follow-

[2] A cubical cell 10^{-2} cm on edge with a specific gravity of 1.0 would weigh 10^{-6} gm and contain roughly 2^{60} molecules of molecular weight 1.0. Since the average molecular weight is clearly greater than 1.0, the actual number of molecules in the cell must be less than 2^{60}.

ing meiosis at autogamy and conjugation. Changes in ploidy fall outside the scope of the present discussion, since they are dealt with in other papers of this symposium. It is important to note that Sonneborn and Schneller (1955a) and Dippell (1955) found that in *Paramecium* such changes are deleterious and appear to be induced secondarily by other unknown hereditary changes associated with the ciliate life cycle.

The Ciliate Life Cycle

The ciliate life cycle has been the subject of investigation for many years by Maupas, Jennings, Woodruff, Sonneborn, and many others (see Jennings, 1944; Sonneborn, 1954). Typically, conjugation is followed by a period of immaturity during which animals cannot mate, then a period of maturity when animals are vigorous and mate readily, and finally a period of senescence during which the fission rate declines and conjugation or autogamy leads to nonviability.

Tens and even hundreds of cell generations may be involved in some cases. The very high degree of stability represented by these changes among the protozoa is better conceived by noting that the complex differentiation of an adult human from a zygote must require an average of only about 40 cell generations. (A single cell weighing 10^{-7} gm would, if all its progeny survived, in only 40 cell divisions produce 2^{40} cells weighing a total of 220 pounds.)

Three major kinds of changes occur during the meiotic cycle. First, in many ciliate stocks there is a sudden loss of the ability to mate following conjugation (but not following the almost identical uniparental process of autogamy!) and a return of the ability after many fissions. Second, in *Paramecium aurelia,* at conjugation and autogamy there is a suppression of the ability to undergo a new autogamy and a return of the ability after approximately 10 to 40 fissions. Third, in many ciliates the suppression of conjugation or autogamy leads, after many cell divisions, to reduced cell division and death, both before and after meiosis. Very little is known concerning the mechanisms of any of these phenomena. Sonneborn and Schneller (1955a) and Dippell (1955) have shown in *P. aurelia* that if aging has not proceeded too far, the postmeiotic nonviability in aged lines results from micronuclear chromosomal aberrations. The aberrations are not accumulated at a constant rate, however,

but appear rapidly in old lines; this suggests that they are induced by the slow change of some inductive system which reaches a threshold. Sonneborn and Schneller (1955b) and Sonneborn *et al.* (1956) have presented evidence against the hypothesis that aging in *P. aurelia* results from the accumulation of chromosomal unbalance in the macronucleus; and Kimball and Gaither (1954) have excluded an accumulation of macronuclear lethals as the causative factor. The ultimate basis for life cycle changes in ciliates is unknown.

Mating Type Determination in Ciliates

At autogamy and conjugation in the ciliated protozoa the macronucleus starts degenerating and the one or more micronuclei undergo meiosis. Two haploid meiotic nuclei fuse to form a diploid syncaryon, which then undergoes a series of mitoses. Some of the mitotic products then differentiate into macronuclei, while others remain micronuclei. At subsequent cell divisions the macronuclei are segregated one to each animal, restoring the vegetative condition. Cytological observations on some ciliates reveal that the diploid number of chromosomes is increased many times in the formation of the macronucleus, and genetic evidence in others confirms this finding. Sonneborn (1937, 1954), working with *Paramecium*, and Nanney and Caughey (1953) with *Tetrahymena* have shown that mating type is determined by the macronucleus, while the macronucleus is itself determined at the time of its formation. Different macronuclear anlagen within the same reorganizing animal may be determined for different types. Environment and genes affect the frequency and range of types determined. In one group of varieties of *P. aurelia* the determination is strongly influenced by the old macronucleus through the cytoplasm so that the new type tends to be the same as the old. The situation resembles cytoplasmic inheritance but differs in that the cytoplasm appears to act only as a vehicle to transfer nuclear influences. The mechanism of the nuclear determinations is unknown, but Nanney (1956) has recently postulated alternative steady-state reactions (similar to those to be discussed presently in connection with antigenic inheritance in *Paramecium*) in the nucleus, sometimes spilling into the cytoplasm.

Self-reproducing Cytoplasmic Particles

Plastids. The inheritance of plastid variations in higher plant forms has been extensively studied. Many cases of variations in form and color of plastids have been shown to be under gene control; others are apparently due to changes in a cytoplasmic genetic system outside the plastids; and others are best interpreted as indicating that the plastid itself is a mutable genetic entity (cf. Rhoades, 1947, for a review). An interesting case in *Euglena* has been studied by Provasoli *et al.* (1951). They have shown that plastids (and in some cases the eyespot) may be destroyed irreversibly and with high frequency by streptomycin. Plastid loss may also be induced by temperature treatments (Pringsheim and Pringsheim, 1952). These findings may be regarded as evidence that the plastid is self-reproducing. On the other hand, it is also possible that the seat of the modified genetic system in *Euglena* is localized outside in the cytoplasm rather than within the plastid.

Kappa and Its Relatives. Kappa is a self-reproducing genetic entity found in the cytoplasm of *Paramecium* and is responsible for the liberation of a toxic substance. Kappa is gene-dependent (Sonneborn, 1943) but mutable (Dippell, 1950). Killers contain several hundred visible Feulgen positive particles, which have been shown to be identical with the genetic kappa particles (Preer, 1950). Mu (μ) particles (Siegel, 1953) are similar to kappa particles except that a toxic action is observed only at conjugation. Pi (π) particles (Hanson, 1954) are kappa mutants that show no toxic action. Other similar Feulgen positive particles (cf. Fauré-Fremiet, 1952) are found in other ciliates. While such bodies appear to be well integrated into the genetics and physiology of their bearers, they nevertheless appear to be highly specialized constituents; they are not general, even in the genus *Paramecium*.

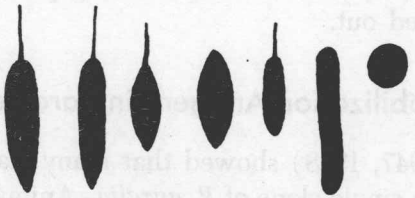
Kinetosomes and Related Structures. Similar bodies play a role in mitosis and give rise to flagella and cilia. In some ciliates they also give rise to the primordia of the trichocysts. Kinetosomes are visibly self-reproducing. Visible self-reproduction, however, does not prove that a structure plays a genetic role in variation. It has been reported that in some forms the kinetosome arises *de novo* (Wilson, 1925, p. 388). However, *de novo* origin does not occur

in others (Lwoff, 1950). Evidence against *de novo* origin of a kinetosomelike body is provided by the finding that chemical treatment of trypanosomes (Piekarski, 1949) induces irreversible loss of the parabasal body. This evidence is not decisive, however, for the site of the mutation may not be within the parabasal body but, instead, elsewhere in the cytoplasm or even in the nucleus.

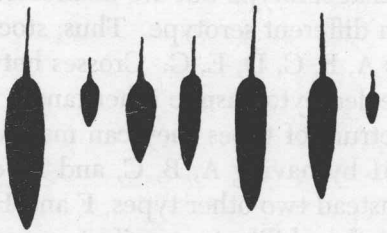
It is of interest to inquire into the inheritance of variations in cilia and trichocysts. If they arise from mutable genetic elements, their mutation would give variants showing cytoplasmic inheritance. The specific mating-type substances of *Paramecium* are found on the cilia (cf. Metz, 1954). However, their variations are under nuclear control (Sonneborn, 1937, 1954a). The immobilization antigens are also found on the cilia in *Paramecium.* As we shall see presently, although both cytoplasm and genes are involved, the assumption of self-reproducing cytoplasmic particles does not help to explain the cytoplasmic and nuclear genetic system established here. We have recently found variant trichocysts within a subline of *P. aurelia,* variety 2, stock 197. The undischarged trichocysts are abnormal and variable in shape, unlike the regular carrotlike form of the original stock 197 or other stocks of paramecia (see Fig. 1). They also differ from the wild type in being very slow to discharge after animals are crushed beneath a cover-slip and observed with the phase microscope. The mutant was stable when selfed and gave a normal F_1 when crossed to normal stock 28 and other normal variety 2 stocks. A backcross to the mutant line gave 13 pairs with both exconjugant clones normal and 12 pairs with both mutant. An F_2 gave 37 pairs with both exconjugant clones normal and 7 pairs with both mutant. Thus both backcross and F_2 results are typical one-factor Mendelian ratios. A few pairs with one exconjugant clone normal and the other exconjugant mutant were found and were presumed to be due to cytogamy or macronuclear regeneration. The results, then, indicate that the abnormal trichocysts are due to mutation at a single locus. It is thus interesting to note that the two known cases of variation in the characteristics of cilia and the first case of trichocyst variation within a variety have provided no evidence for a genetic role of these structures or their primordia. More cases are needed.

Mitochondria. Cytological observations show that mitochondria often divide, but they also appear to fuse and disintegrate; there exist claims that they arise *de novo* as well. Their resemblance to plastid primordia has been cited as evidence that they, like plastids,

may be self-reproducing. The best, but still only suggestive, evidence for their genetic role comes from the finding that cytochrome-deficient mutants in yeast and *Neurospora* are often cytoplasmically inherited (Ephrussi, 1953; Mitchell *et al.*, 1952, 1953). The cytochromes, of course, are known to be localized in the mitochondria of higher forms. No variant mitochondria have been reported in the protozoa; but genetic analysis of such variants, provided they can be found, would appear to be our best hope of ascertaining whether mitochondria have a genetic role.



STOCK 197



STOCK 28

Fig. 1. Sketches of the undischarged trichocysts from the aberrant stock 197 of *Paramecium aurelia* are shown in the upper row. Those of normal stock 28 are shown in the bottom row.

The Gullet. Conjugants of paramecia may be induced by antiserum to fuse, permanently but incompletely. Such doubles produce fairly stable clones of individuals with a double form containing two gullets, and one macronucleus (Sonneborn, 1950a; Margolin, 1954). These facts are most reasonably interpreted by assuming that the hereditary basis for the double condition lies within the cytoplasm and not in the nucleus. The question arises as to whether the whole cytoplasmic organization is responsible for the form or whether the mechanism can be reduced to one or more individual elements. Cytological observation might possibly suggest that the gullet is self-reproducing. Perhaps the double form is determined

and maintained by the organization of the cytoplasm into the double form by the two self-reproducing gullets. Hanson (1955) has attempted to study the question by irradiating one of the two gullets in double animals with an ultraviolet microbeam. A double animal, with gullet damage so extensive that no visible irradiated gullet remains, may regenerate a new gullet, but more often it reorganizes as a single animal with one gullet—a process that sometimes occurs spontaneously. The interpretation is not clear, but the results show that a complete gullet is not necessary for a new gullet. The possibility that the gullet has a self-reproducing primordium, however, has not been ruled out.

Immobilization Antigens in *Paramecium*

Sonneborn (1947, 1948) showed that many stable serotypes can be isolated from a single clone of *P. aurelia.* Animals of a given serotype are immobilized when placed into an appropriate dilution of antiserum against that serotype but are unaffected when placed into antiserum against a different serotype. Thus, stock 51 yielded stable clones of serotypes A, B, C, D, E, G. Crosses between the serotypes within a stock revealed cytoplasmic inheritance. Stocks were found to vary in the spectrum of types they can manifest. Thus stock 29, which resembles 51 by having A, B, C, and D, did not manifest E or G but showed instead two other types, F and H. Crosses between stocks showed that the ability to manifest any given type, such as F, is due to a single Mendelian gene. Furthermore, in comparing different stocks, specific differences in serotypes, such as 29A and 51A, are often found. Such differences are also controlled by single alleles, one locus for each serotype. This picture, originally established for variety 4 of *P. aurelia,* has been confirmed and extended to several of the other varieties (cf. Beale, 1952; Finger, 1957a, 1957b; Pringle, 1956). Transformations from one serotype to another within a stock occur spontaneously but may also be induced by specific antiserum, radiation, changes in nutrition, temperature, and various chemical treatments.

The general situation may be summarized as follows: each organism contains a number of specificity determining loci, each concerned with one of the different serotypes. The specificity locus has, in the one case thus far investigated, been shown to be the same as the locus determining presence and absence (Reisner, 1955).

Usually one locus may come to expression at a time, i.e., only one serotype is manifested at a time. In most of the cases studied, any one of several different loci may come to expression under any one given set of environmental conditions, but once expressed, the continued expression of that locus, rather than other loci, is cytoplasmically inherited. The tendency to shift from the expression of one locus to a new one (transformation) is conditioned by this system of cytoplasmic inheritance, by the environment, the specificity locus and other loci (Beale, 1954).

Recently, studies on the immobilization antigens have been made by means of specific precipitation in gel (cf. Oudin, 1952). A technique of double diffusion in agar, studied quantitatively by Preer (1956), has been used by several workers. Antiserum is placed into the bottom of a small tube; agar is layered on top; and antigen is added on top of the agar. The antigens and antibodies diffuse into the agar, forming bands of precipitation where they meet, each separate antigen-antibody system generally forming a separate band. Relative concentrations of antigen and antibody may be found by taking advantage of the fact that the band position, p (the distance from antigen-agar interface to the band, divided by the total agar length), is linearly related to the logarithm of the ratio of the concentrations of antigen and antibody. By setting serial dilutions of antigen against constant antibody concentration, one obtains a linear relation that permits quantitative estimates of the antigen (see Fig. 2). Furthermore, information concerning diffusion

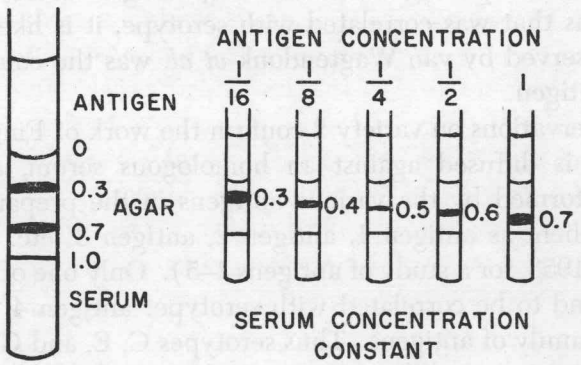


Fig. 2. A double diffusion tube is sketched on the left, showing band position, p, for bands at 0.3 and 0.7. The series of tubes sketched on the right shows the change in p obtained when serial dilutions of antigen are set against a constant antiserum concentration.

coefficients may also be obtained, for Ouchterlony (see Preer, 1956) showed that when the antigen and antibody are mixed in their immunological equivalence ratio, a stationary band is formed, and that

$$\frac{p}{1-p} = \sqrt{\frac{D_{ag}}{D_{ab}}}$$

where D_{ag} and D_{ab} are the diffusion coefficients of antigen and antibody respectively.

Finger (1956), using this technique, found that many antigens may be demonstrated in extracts of homogenates and of lyophilized animals of variety 2, serotype G. Extracts of serotype C had all these antigens but one, and in its place had another specifically different antigen. Thus each of the two serotypes was found to have a unique serotype-correlated antigen, for all the other antigens detected in animals of the two serotypes appeared identical. The almost perfect correlation between the antibody corresponding to these antigens and the presence of immobilizing activity of a large series of antisera against various serotypes leaves no doubt that he was working with the immobilization antigen or a closely associated substance.

Van Wagtendonk *et al.* (1956) report that if animals of variety 4 of *P. aurelia* are placed into 0.06M saline, a soluble antigen is liberated, which can be shown by the Oudin agar diffusion test to react with homologous and not heterologous antisera. Since the antigen studied by Finger was the *only* antigen detectable in his preparations that was correlated with serotype, it is likely that the antigen observed by van Wagtendonk *et al.* was the counterpart of Finger's antigen.

Our observations on variety 2 confirm the work of Finger. When an extract is diffused against an homologous serum, a series of bands are formed by the various antigens in the preparation. We designate them as antigen 1, antigen 2, antigen 3, etc. (see Preer and Preer, 1958, for a study of antigens 1–5). Only one of these antigens is found to be correlated with serotype: antigen 4. Antigen 4 is really a family of antigens. Thus serotypes C, E, and G have been found to contain a specific antigen 4 lacking in the others. We distinguish the different kinds of antigen 4 by subscripts, e.g., 4_C, 4_E, 4_G. The different antigen 4's are closely related, being present in approximately the same structures, in the same quantities, and having