CELLULAR MECHANISMS IN DIFFERENTIATION AND GROWTH

THE FOURTEENTH SYMPOSIUM OF
THE SOCIETY FOR THE STUDY OF
DEVELOPMENT AND GROWTH

Executive Committee, 1955

V. C. Twitty, Stanford University, *President*
M. V. Edds, Jr., Brown University, *Secretary*
R. W. Briggs, Lankenau Hospital Research Institute, Philadelphia, *Treasurer*
J. T. Benner, Princeton University
Harriet B. Creighton, Wellesley College
D. R. Goddard, University of Pennsylvania

Cellular Mechanisms in Differentiation and Growth

M. DELBRÜCK AND W. REICHARDT · I. MANTON
HEWSON SWIFT, LIONEL REBHUN, ELLEN RASCH AND
JOHN WOODARD · JOSHUA LEDERBERG AND
ESTHER M. LEDERBERG · VANCE TARTAR
M. C. NIU · RALPH H. WETMORE · BETTY BEN GEREN
VIKTOR HAMBURGER · R. E. BILLINGHAM
MAURICE SUSSMAN AND RAQUEL R. SUSSMAN

EDITED BY DOROTHEA RUDNICK

PRINCETON, NEW JERSEY
PRINCETON UNIVERSITY PRESS
1956

L. C. CARD 56-10678

Printed in the United States of America by
The Vail-Ballou Press, Binghamton, N.Y.

FOREWORD

Wir . . . machen die Entfaltung der Anlagen abhähgig von Bedingungen oder Ursachen, die ausserhalb der Anlagesubstanz . . . liegen, aber trotzdem in gesetzmässiger Folge durch den Entwicklungsprocess producirt werden . . . in den Wechselbeziehungen, in welche die Zellen eines Organismus, während sie durch Theilung an Zahl zunehmen, in einer sich stetig verändernden Weise zu einander treten. . . .

OSCAR HERTWIG

THE Society for the Study of Development and Growth held its fourteenth Symposium at Amherst College on June 15–18, 1955, and may again congratulate itself on demonstrating that constructive and leisurely scientific communication is possible in the middle of the twentieth century. The Local Committee at Amherst, adept in this variety of sorcery, produced fine weather, material comfort, and an atmosphere of warm friendliness. The Society is deeply indebted to all those individuals who made the meeting so successful, as well as to the National Science Foundation which generously subsidized it.

The Symposium speakers, among their varied interests and approaches to the problems of growth and differentiation, appeared to have a common focus on the cellular aspects of these problems. Their contributions have accordingly been grouped on the basis of whether the focus was on events and structure within a chosen cell, or on interrelations of differing cells.

In the first series of chapters, the authors study localized events in single cells with refined tools of a wide variety: rigorous physicomathematical analysis, spectrophotometric and electron microscopy, and microsurgery. Thus the pioneering biophysical investigation of Delbrück and Reichardt develops manageable methods of expression for the conditions of elongation in the sporangiophore of a mold. Swift and his collaborators give quantitative data on changes in localized depots of ribonucleic acid as between nucleus and cytoplasm of cells. Manton demonstrates a remarkably stereotyped geometry in the microstructure of cilia throughout plant and animal kingdoms; Tartar analyzes a cortical pattern-system in a ciliated protozoon.

Relations between differing cells offer, in multicellular systems, an almost limitless field for investigation of mechanics of development. Of these relations, one of the most basic and puzzling is that of the fusion

of differing gametes in the sexual life-cycle. From a wealth of experimental information, which already has transformed (or transduced) biological thinking on the subject, Lederberg and Lederberg discuss genetic aspects of sexual and infective phenomena in bacteria.

To advert to ontogeny of many-celled systems, it has long been apparent that once even two physiologically different types of cell are present, one of these may further affect the other either by contact or more remotely by production of diffusible substances. Sussman and Sussman find evidence even in slime molds for physiological differentiation as well as for mutual influence between cells in their studies of synergy in mutant strains. The classic two-tissue relationship in vertebrate animals, that of the induction of the Amphibian nervous system by organizer, is shown by Niu to be mediated by diffusible substances operating at the cellular level; advances in the characterization of these substances have been made. In higher plants, Wetmore emphasizes the control of growth and differentiation of axial systems by the auxins. Students of plant development are fortunate in having fairly complete knowledge of the biochemical characteristics and physiological action of these substances.

Maturing, rather than preliminary, stages in cell and tissue differentiation in higher animals, likewise, offer many situations where interrelations between two sorts of cell can be analyzed. For the case of the vertebrate nervous system, Hamburger reviews an array of problems, involving on one hand contact effects, as in the control of pattern in the central nervous system by the periphery through neurons, and on the other hand a diffusible agent, probably protein in nature, stimulating development of specified ganglionic neurons. Geren shows by electron microscopy a new, specific, and strikingly geometric cell-to-cell relation in the differentiation of the myelin sheath. Finally, for integumentary tissues and blood, Billingham advances knowledge of the differentiation and control of specificity by decisive experiments on higher vertebrates.

Yale University DOROTHEA RUDNICK

CONTENTS

Foreword v

I. INTRACELLULAR EVENTS

I. System Analysis for the Light Growth Reactions of Phycomyces 3
By M. Delbrück and W. Reichardt

II. The Cytology of Nuclear RNA 45
By Hewson Swift, Lionel Rebhun, Ellen Rasch and John Woodard

III. Plant Cilia and Associated Organelles 61
By I. Manton

IV. Pattern and Substance in Stentor 73
By Vance Tartar

II. INTERCELLULAR RELATIONS

V. Infection and Heredity 101
By Joshua Lederberg and Esther M. Lederberg

VI. Cellular Interactions during the Development of the Cellular Slime Molds 125
By Maurice Sussman and Raquel R. Sussman

VII. New Approaches to the Problem of Embryonic Induction 155
By M. C. Niu

VIII. Growth and Development in the Shoot System of Plants 173
By Ralph H. Wetmore

IX. Developmental Correlations in Neurogenesis 191
By Viktor Hamburger

X. Structural Studies of the Formation of the Myelin Sheath in Peripheral Nerve Fibers 213
By Betty Ben Geren

XI. Acquired Tolerance of Foreign Cells 221
By R. E. Billingham

Index 233

CELLULAR MECHANISMS IN DIFFERENTIATION AND GROWTH

I. SYSTEM ANALYSIS FOR THE LIGHT GROWTH REACTIONS OF PHYCOMYCES

BY M. DELBRÜCK AND W. REICHARDT [1]

Living organisms have evolved complicated chains of action which serve to gather information from the environment, to correlate and to evaluate this information, and to react to it. In some higher organisms these three functions may be assigned to anatomically distinct structures: the sensory apparatuses, the central nervous system, and the effector apparatuses. This crude schematization is, of course, somewhat arbitrary. The chains of action are often circular in that an effector (a vertebrate muscle spindle fiber, for instance) may at the same time serve as a sense organ. In fact, what we call the stimulus, and what we call the response depends on our point of view. The impulses conducted in an afferent fiber constitute the reaction to the stimulus which has fallen on the sensory element, and at the same time they constitute the input, or stimulus, which carries information to the next stage. The subdivision can be carried further, to the individual cell where we might in principle make the same distinction between input, evaluation, and output. In lower organisms, in fact, we may have no anatomical separation at all, or at least not an apparent one, and yet even on these lowest levels we find realized some of the features which might seem to require a high organizational complexity. Thus, the feature of adaptation to various levels of stimulus intensity, so characteristic of every one of the sense organs of higher organisms, is also found in the lowest forms. The mechanisms by which the adjustment to various ranges of stimulus intensity is accomplished may differ greatly from one organism to another. At present we do not have an understanding of the mechanism for any one case. The reasons for this are well known to those familiar with the problems of sensory physiology. For the most part they are connected with the fact that the systems to be studied are multicomponent systems and that the experimenter encounters great difficulties in isolating these components

[1] California Institute of Technology. This work was supported by a grant from the National Science Foundation. The senior author wishes to acknowledge the help he has received during the earlier stages of this work at various periods from Dr. Mavis Middlebrook, Dr. Alex Keynan, and Dr. René Cohen.

in such a form that their separate functions can be studied. Before turning to the subject of our own experimental work, we would like to offer, by way of justification for our choice of material, some general remarks about stimulus-reaction systems.

I. THRESHOLD SYSTEMS VERSUS GRADED SYSTEMS

The stimulus-reaction systems which have been studied most intensively by physiologists during the last hundred years are *threshold* systems, or so called all-or-none systems. They show no obvious reaction for stimuli below the threshold, and a unique reaction when the stimulus is above the threshold and the system gets excited. Let us consider the nerve fiber, a system most extensively studied and most deeply analysed. When a fiber is stimulated in one region and the response tested in a distant region, the system is indeed an all-or-none system. We either do or do not get the conduction of an impulse and the characteristics of the impulse are independent of those of the stimulus. The situation is somewhat different if the response is tested not in a distant region but in the immediate vicinity of the stimulus. Here we find that stimuli below threshold do produce a small response, the local response. For stimuli below threshold the response increases at first more or less proportionally with the stimulus, and is still small compared to full excitation for stimuli just below threshold. As we approach threshold the responses increase disproportionately. As we exceed threshold the character of the local response abruptly becomes regenerative and increases to a maximum size. The local response gives rise to a conducted impulse only if it has this large size. Thus the system which, with respect to the distant test, had an all-or-none character, could be described as having an all-or-some character with respect to the local test. The notion of threshold is applicable also in this latter situation.

The all-or-none character of the conducted impulse for many years held a peculiar fascination for physiologists. We believe that this was so not only because of its easy access to experimentation, but also because it was felt that it lent itself particularly well to the scientific ideal of objectiveness. A response which is either there or not there is obviously much less subject to errors of observation and to subjective deception than anything that has to be measured quantitatively. It has an appealing simplicity which easily deludes one into the belief that the underlying mechanism must be simple too. Of course it was realized that there must always exist a finite local response, even before it has

built up to full excitation. However, it was felt that studies which relate stimuli with all-or-none reactions should be fruitful for inferring the mechanism of the local response. Numerous were the theories aiming at this goal, and they all involved a number of imaginary time-dependent variables, supposedly characterizing the instantaneous state of the stimulated region. The link between these hypothetical variables (degree of excitation and refractoriness, for instance) and the observable reaction was established by postulating that threshold is reached when one of these variables exceeds the other. Thus, with respect to the hypothetical local response, the experiments test only one point of the postulated functional relations. In recent years the inadequacy of this procedure has become very apparent, and great efforts have been made to study the local reaction as such. Only very recently have these attempts met with any degree of success. The principal difficulty has been that of devising procedures which would permit a meaningful quantification of the stimulus and of the response. To accomplish this it is necessary to design the stimulus and the apparatus picking up the response in a manner concordant with the geometry of the system. In other words, the stimulating electrodes and the pick-up electrodes have to be cylindrical and co-axial with the system. Only in this situation do the measured potentials and currents give an indication of meaningful variables. Hodgkin, Huxley, and Katz (1952) have carried out such an analysis, and their studies demonstrate the extraordinary complexity of the local response. Chemical and electrical changes appear to be intertwined in a most intricate manner and the mechanisms of these chains of action are as yet quite obscure. It appears that Nature had to go to enormous length to design a system having threshold characteristics.

This finding is not surprising if one has some familiarity with corresponding engineering problems. If the input and output variables of such a system are by their nature continuous variables, the discreteness characteristic of threshold relations can be brought into the system only by introducing singularities into their functional relations. This means, above all, that the relations must be non-linear, and therefore, enormously more difficult to analyse than are linear ones. Thus we believe that the preoccupation of physiologists with all-or-none systems had its motive in a two-fold delusion. On the one hand, we have the thought that the unambiguousness of the all-or-none reaction presents a particular advantage. From an experimental point of view this advantage is enormously outweighed by the disadvantage that the obser-

vations characterize only a single point in the primary stimulus response diagram and that no real progress is made until this primary system is made the object of direct experimental investigation. On the other hand, such systems, far from being simple, must in fact be particularly involved from the point of view of the functional relations between the variables involved in the primary response. A relative simplicity may only be expected considerably below threshold where the system might be idealized by a linear one. Thus the most useful region from the point of view of holding out hope for a real understanding of the underlying mechanisms is particularly narrow just for these systems and involves particularly small responses. We believe, therefore, that threshold systems represent a very poor choice, as far as simplicity goes, and much more effort should be made to study graded systems. Now it is true that the primary sensory elements in higher organisms are believed to represent such graded systems. Almost invariably, however, the evidence for this is very indirect. For instance, in the case of the muscle spindles the gradedness is inferred from measurements of the impulse pattern in the attached sensory fiber, and thus is deduced only after the hypothetical graded response has been converted into a pattern of all-or-none responses in a secondary system. Or, in the case of the vertebrate eye, we have the electro-retinogram, seemingly a graded response, which is believed to be a direct reflection of the responses of the primary receptors, averaged over large numbers of these. There seems to be no hope, however, of carrying the analysis to the level of the individual receptor. Most direct is the evidence, perhaps, for the Limulus eye, where the analogue of the electro-retinogram can be picked up from a single cell of an ommatidium (Hartline et al., 1952). In this system, however, the geometry and the anatomy of the elements involved are highly unfavorable, and considerable doubt attaches to the question which of the elements of the ommatidium are to be considered as primary receptors. We will have more to say about this system later on.

In the search for a material more amenable to a thorough quantitative experimental study of the relations between stimulus and reaction, and involving a very pronounced range adjustment mechanism, we were attracted by the light growth reactions of the sporangiophores of the mold Phycomyces. This system has been studied off and on for almost 100 years, but we believe that its potentialities have never been fully exploited. Its peculiar advantages will become clear, we hope, from the description which follows.

II. MATERIAL AND METHODS

Our organism was a strain of *Phycomyces blakesleeanus* (strain 1555 of the National Regional Research Laboratory). It was grown on autoclaved potato slices in aluminum thimbles (test tube caps), 15 mm in diameter, 20 mm high. For inoculation the spores were heat shocked for 30 minutes at 45°C to insure good germination. Each pot was inoculated with only a small number of spores. This procedure appears to give each spore a chance to form a large mycelium and thereby to produce stronger sporangiophores. The sporangiophores come up three days after inoculation, and a pot can then be used for about a week thereafter producing a fresh crop of sporangiophores every morning after being cut back the preceding evening.

Experiments were conducted in a dark room in which the temperature varied between 25° and 26° over the course of days, but never more than a fraction of a degree during any one run. The pots were mounted on a tray attached to a mechanical micromanipulator permitting fine motions in three directions. Growth was measured with a Zeiss-Opton binocular microscope and ocular micrometer at a magnification 80. One scale division of the micrometer equals .025 mm. Readings were taken to ⅒ of a scale division. The readings were taken in red light of a purity to give no phototropic effect in several hours of exposure.

The stimulating light source was a 500 watt projection bulb, air cooled, mounted in a vertical opaque cylindrical housing with two portholes containing lenses of 5 cm focal length. The light paths from here to the specimen are illustrated in Fig. 1. The illumination reached the specimen from the right and left, and coming down at an angle of 30° with the horizontal, for reasons of stabilization, as explained in the section on phototropism. The lenses were slightly defocused so as to produce a fairly uniformly illuminated spot of about 2-cm diameter at the specimen. The light was filtered through Corning 5-61 light filters. These filters transmit between 380 and 480 mμ, with a maximum transmission at 425 mμ. This transmission curve is very similar to the phototropic action spectrum as determined by Castle (1931). In addition to these color filters, there was provision for inserting glass plates to balance minor inequalities between the illuminations from the right and from the left. The light source was powered from the 115 volt net via a voltage stabilizer. Its intensity was regulated with Variacs. Various periodic illumination programs could be arranged with the help of

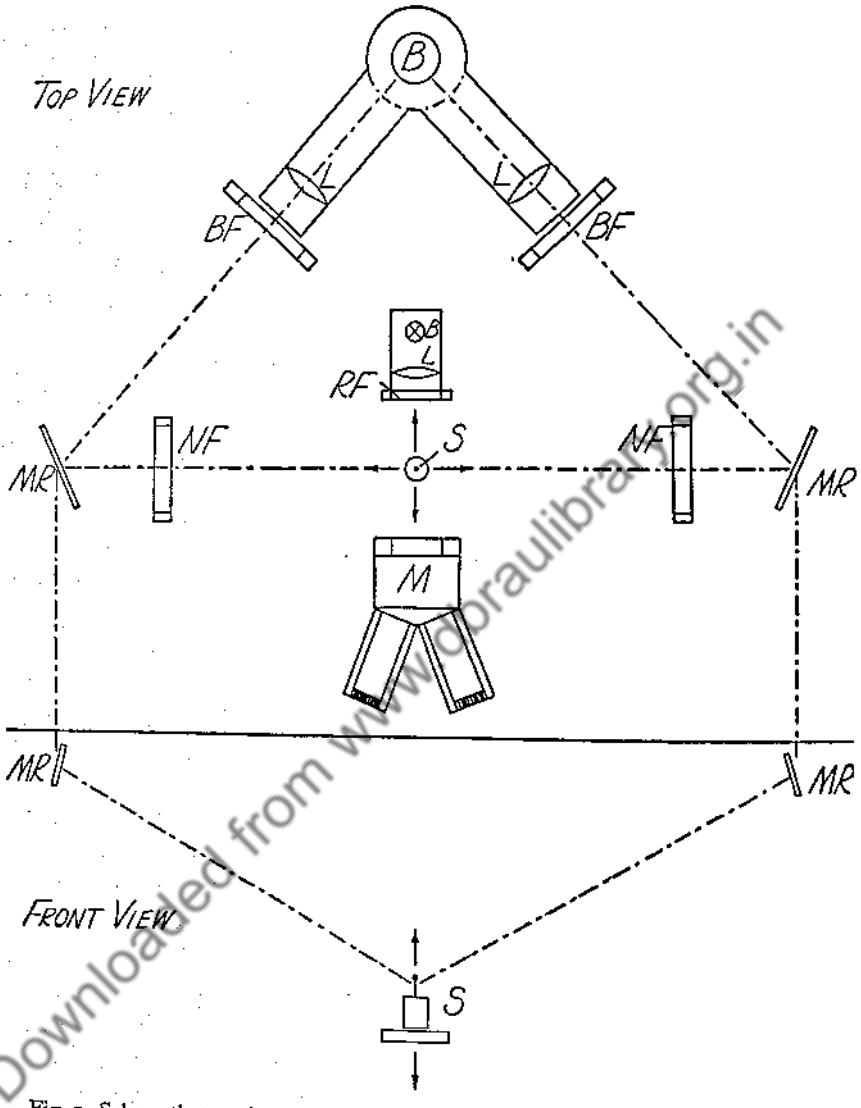


Fig. 1. Schematic top view and front view of the illumination set-up. *B*, light sources; *L*, lenses; *BF*, *RF*, *NF*, blue, red and neutral filters; *Mr*, mirrors; *S*, specimen; *M*, binocular microscope. The arrows at the specimen indicate the directions of displacement produced by the mechanical micromanipulator.

Flexipulse time switches and relays. The intensities were calibrated with the help of a convenient phototube photometer designed by Mr. George Bowen. The intensities given in this paper will be given in terms of a unit intensity corresponding to an energy flux at the specimen of about 100 erg/cm² sec. In our diagrams the intensities are given

on a logarithmic scale, the logarithm being taken to the base two. Thus, $\log I = -10$ means that the intensity was 2^{10} times smaller than our unit intensity, or about .1 erg/cm² sec. For purposes of comparison with earlier work, where white light was used, rated in meter candles, one should figure 1 MC = 1.6 erg/cm² sec (Westphal, 1947). The effectiveness of our blue light is about four times greater, per erg, than that of unfiltered light. Therefore, our unit intensity equals in effectiveness about 250 MC. At maximum dark adaptation a stimulus size of 2^{-15} ($I = 2^{-13}$, t = .25 min) was the lowest that would give a measurable growth response. This corresponds to about .5 MCS. On continuous unilateral illumination tropic responses were obtained down to $I = 2^{-20} = .00025$ MC.

III. GENERAL CHARACTERISTICS OF THE GROWTH OF THE SPORANGIOPHORES OF PHYCOMYCES

Phycomyces grows well on a variety of media, including chemically well-defined synthetic media. Vegetative spores inoculated into such a medium produce a dense mycelium, and after a few days sporangiophores are formed. These grow straight up to a height of about 1 cm and then form a spherical sporangium at the tip. During the formation of the sporangium growth in height is arrested. A few hours after the sporangium is fully formed growth in height resumes, and within a few hours a steady growth rate is attained of about 3 mm per hour. This steady growth rate is maintained for many hours. This is stage IVb of the classifications of Errera (1884) and Castle (1942). It is this stage we will be principally concerned with. The sporangiophore is a hollow tube filled with protoplasm and a vacuole. It is about .05 mm in diameter. The growth is confined to a growing zone about 2 mm long and located immediately below the sporangium. Below this zone the wall of the tube is secondary wall. Here the wall grows in thickness, but not in area. In the growing zone the wall is exceedingly thin and its growth consists of stretching in the vertical direction. The growing zone stretches without getting longer: for every amount that it grows in length a corresponding amount at its bottom is converted into secondary wall which has ceased to grow in length. Similarly, the wall in the growing zone, as it stretches, does not become thinner: for every amount that it becomes thinner new wall material is interpolated. The principal layer of the wall in the growing zone appears to consist of chitin microfibrils about 200Å in diameter, arranged more or less horizontally (Frey-Wyssling, et al., 1950). As the wall stretches these mi-

crofibrils separate from each other, and new fibrils are laid down to fill the gaps. Thus, a steady state is maintained for many hours (Fig. 2A).

Oort (1931) discovered another feature of this growth termed by him "spiral growth." It becomes obvious when small particles attached to the sporangium or to the growing zone are watched closely during

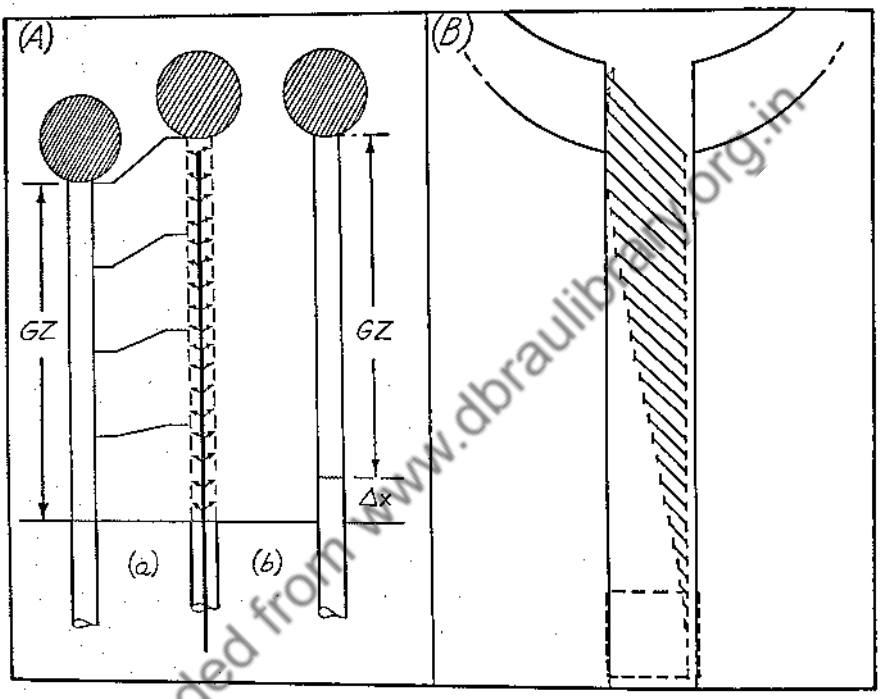


Fig. 2. Two schematic diagrams of the growing zone, illustrating the kinematics of growth. (A) illustrates the steady state: the growing zone, GZ, is shown at the left, and again at the right displaced by an amount Δx. The picture in the middle decomposes this continuous process into two partial processes. In process (a) the growing zone is stretched, separating the micro-fibrils in the wall. In process (b) these gaps are filled by the interpolation of new fibrils and the bottom section of length Δx is converted into secondary wall.

(B) illustrates spiral growth. The position of a row of markers, vertical at time zero, is shown again after 10 min. During this interval the growing zone has moved up by ⅓ of its length. The top marker has made one half of a revolution, intermediate markers have rotated less. Their vertical displacement is correspondingly smaller, so that the tilts of the directions of motion of the markers are alike.

growth. Such observations show that the sporangium and each section of the growing zone rotate around the vertical axis during growth. The rotational velocity is fastest for the sporangium, and decreases to zero as the bottom of the growing zone is approached. In a typical case a

sporangium makes a full revolution in about 20 minutes. The path of any point on the sporangium or on the growing zone during growth is thus not a straight line, but a helix, a left-handed helix during the stage we are considering (Fig. 2B). For a point just below the sporangium the pitch of this helix, i.e. the angle which a tangent makes with the vertical, is about 10°. This helical growth is very obvious when one knows about it, because one can hardly avoid having occasional dust particles adhering to the sporangium, and these particles can be seen in the microscope to alternate between the right and left side with perfect regularity. It is truly astonishing that this phenomenon was not noticed by the several very careful observers who preceded Oort.

IV. THE LIGHT-GROWTH REACTION

The sporangiophores of Phycomyces are positively phototropic. When they are exposed to light from one side they grow towards the light. Blaauw (1914) discovered another effect of light on the growth of the sporangiophores, which is related to the phototropic response in a manner as yet only partially understood, and about which we will have more to say presently. Blaauw's effect, the *light-growth response,* refers to a situation in which the specimen is at all times symmetrically illuminated from two or more sides. If the illumination is symmetric with respect to the vertical axis, and if the specimen is growing vertically at the start of the experiment, this illumination will not cause it to deviate from vertical growth. In fact, if illumination is kept at a constant intensity for a certain length of time, the rate of growth is also constant and *is the same whatever the intensity.*[2] However, a striking and transient change in growth rate occurs when the intensity of illumination is changed. Fig. 3 illustrates the growth responses to four basic illumination programs. Let us consider here only the first of these, the "pulse-up" program. The specimen is exposed to a short period of illumination with a higher intensity than that to which it had previously been adapted. After the stimulus growth continues at its normal rate for 2.5 minutes, then increases for a few minutes to a maximum which may be twice as high as the normal rate. Presently it decreases again, goes below normal, and returns to normal by about 15

[2] Blaauw (1918) and Tollenaar and Blaauw (1921) find a very slight dependence of the growth rate, in the stationary state, on the intensity of illumination. We have not been able to confirm this observation. The effect is a difficult one to establish since the growth rate, even under the most uniform conditions, is subject to slow variations of the same order as those claimed by the cited authors.

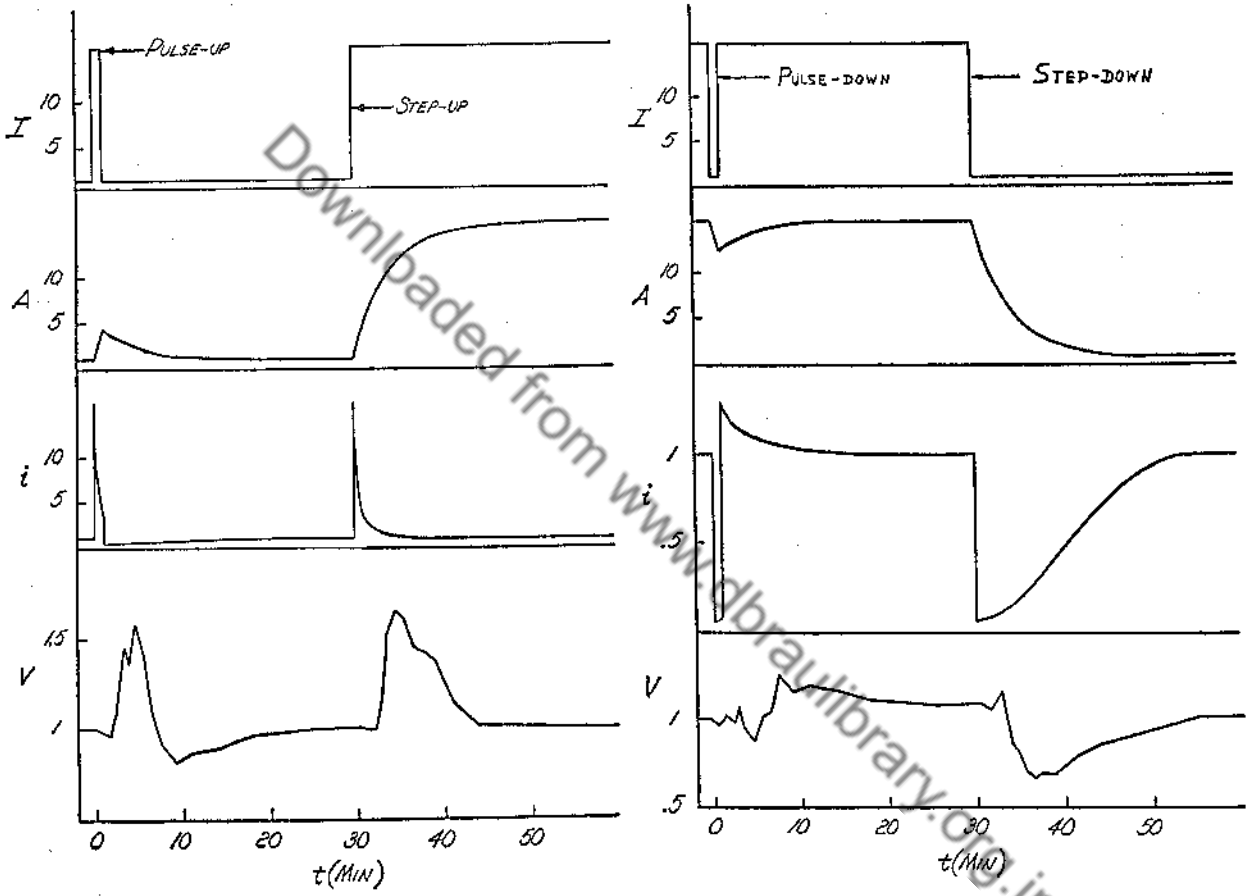


Fig. 3. Four basic illumination programs and their outputs. The top row gives the program, intensity I (linear scale) versus time t. The bottom row gives the growth output, velocity v (relative to average velocity) versus time. The four programs were run consecutively, with 30 min intervals, as indicated in the top row. One full cycle thus takes two hours. The whole cycle was repeated four times and the results averaged. All measurements on one specimen.

The second and third row give the level of adaptation, A, and the subjective intensity, $i = I/A$, calculated according to the theory developed later (equation 1b). Note that the scale used to plot $i(t)$ is twenty times larger for the "down" than for the "up" programs.

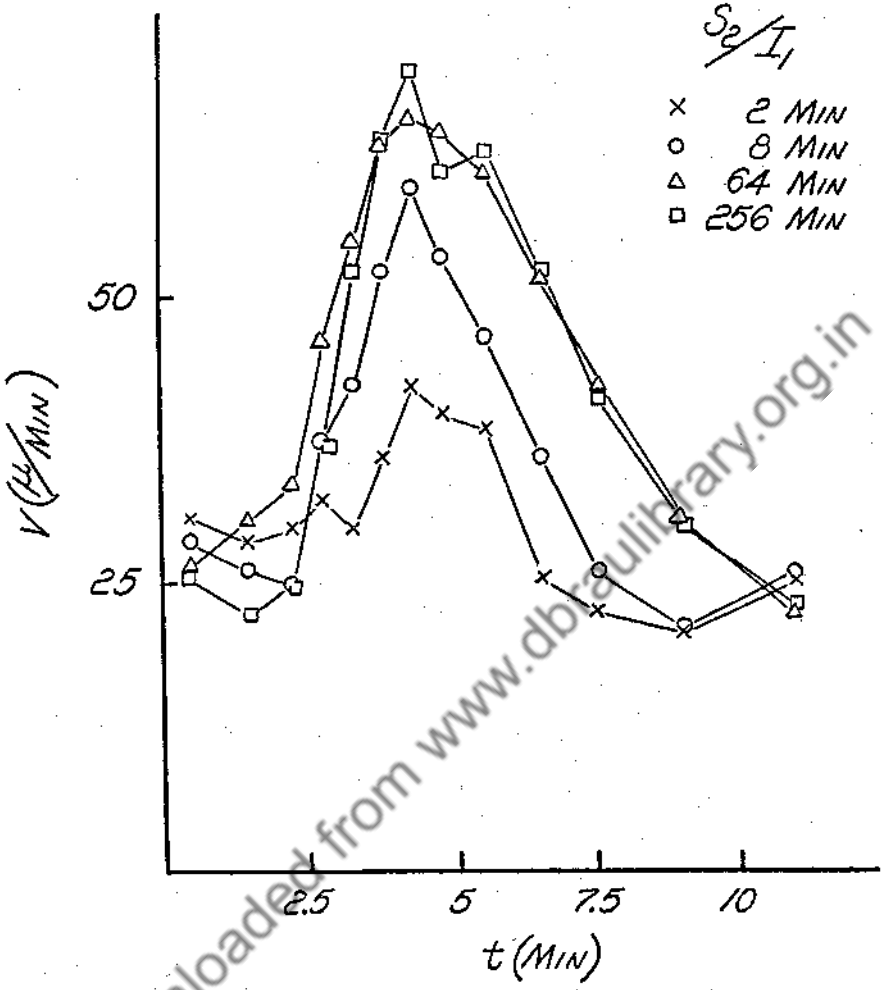


Fig. 4. Growth velocity v versus time t for various values of S_2/I_1. The specimens were equilibrated with $I_1 = 2^{-3}$. Stimuli were given at 20 min intervals. The response curve for each stimulus was measured four times and averaged. Duration of each stimulus was .25 min.

minutes after the stimulus.[3] The net gain in growth due to such a stimulus is zero, i.e. the transient increase in growth rate is compensated for by the subsequent fall below the normal level.[4] The stimulus does

[3] Oort (1932) finds that the positive phase of the response has several maxima and minima, following each other at intervals of less than a minute. We have not been able to confirm this observation. Blaauw (1918) finds that the negative phase of the response is modulated by slow, damped oscillations. This we were also unable to confirm.

[4] There are conflicting reports in the literature as to whether the growth response does or does not involve a net gain in growth. Experimentally the question is difficult

not produce extra growth, it simply alters the *distribution in time* of the growth that would have taken place during the same period in the absence of the stimulus. The illumination program controls the rate of utilization of materials available for growth, but does not alter the quantity of this material.

If we repeat this experiment, keeping the adapting intensity constant, but varying the intensity or duration of the stimulus, we find first of all that the reaction is a graded one. With decreasing stimulus the response changes gradually until it becomes too small to be picked up by the observer (Fig. 4). Secondly, we find that a change in the stimulus does not alter the latent period.[5] This remains always close to 2.5 minutes. Thirdly, we find that the shape of the response curve is independent of the stimulus, except for very large stimuli, for which the positive phase of the response may be a little and the negative phase may be much lengthened. Fourth, we find that the response depends on the product intensity $\times$ time as long as the duration of the stimulus does not exceed, say, one minute (Fig. 5).

The other three basic programs illustrated in Fig. 3 give quite different responses. The step-up program shows only a positive phase in its growth response, thus involving a net gain in growth. The step-down program gives only a negative response which is shallower and of longer duration than the response to the step-up. The pulse-down program gives a very slight response, consisting apparently of a short negative phase followed by a shallow positive phase of long duration.

V. THE PHOTOTROPIC RESPONSE AND THE LIGHT GROWTH RESPONSE

We have mentioned that the sporangiophores of Phycomyces are phototropic. We would like to insert here a discussion about what we know and what we do not know about the interrelationship between

to decide because of the uncontrollable fluctuations cited in footnote 2. We have compared the *average* growth rate during a series of periodic stimulations (period 5, 10, 15 minutes) with the average growth rate during constant illumination. If there is a net gain in growth for every stimulus which produces a growth response this method should bring it out strongly. However, no effect was found.

[5] Castle (1929, 1930) and Castle and Honeyman (1933) report detailed studies of the dependence of the latent period on the stimulus size. We have not been able to confirm these results. The measurements depend on fixing the moment of beginning of a change in growth rate. In practice that means that one fixes the point at which the change in growth rate exceeds the error of measurement. This occurs earlier when the rate changes faster, even if the true latent period is constant. Measurements of this type should, therefore, not be taken at their face value as indicating changes in the latent period.

these two responses. It should be noted that for the plant the tropic response is the important one. The growth response as such does not serve any obvious purpose in the life of the plant. The tropic response does not serve, as in higher plants, the purpose of seeking light, but

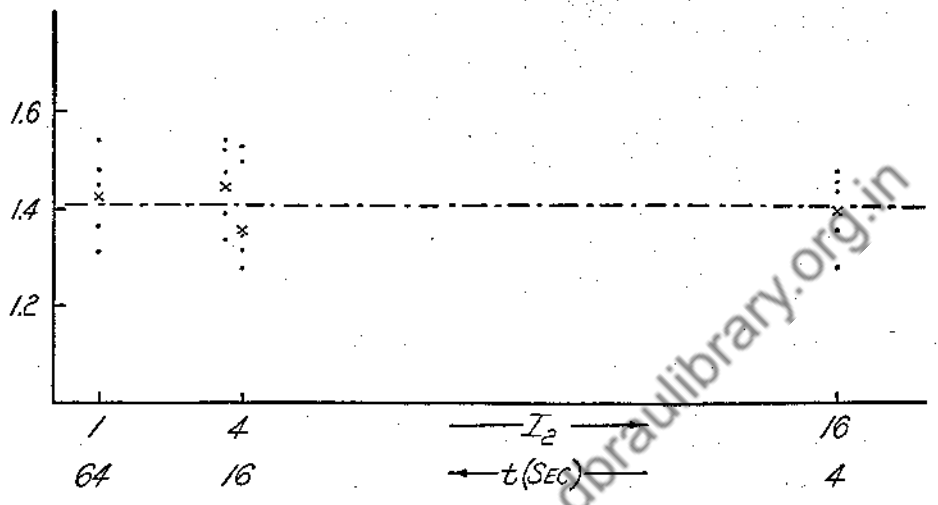


Fig. 5. Reciprocity between time and intensity. Two experiments, each involving one specimen. In the first experiment stimuli S_2 and S'_2, in the second experiment S'_2 and S''_2 were compared, where

	t_2	I_2	$I \times t$
S_2	4 sec	16	64
S'_2	16 sec	4	64
S''_2	64 sec	1	64

In each experiment the two types of stimuli were given alternately at 20 min intervals, each five times. The individual results are represented by dots, the averages by crosses. The dot-dash horizontal line indicates the average of all responses. Equilibrating intensity $I_1 = 2^{-3}$.

that of seeking "the open" so the spores can be widely dispersed. The light is used as a pilot to find this open space. The tropic and the growth responses are obviously closely related.[6] They have the same latent

[6] In the older literature the phototropic response is occasionally spoken of as an all-or-none response and experiments are reported in which the "proportion of responding sporangiophores" is listed as a function of the illumination program. Also, a great deal of variability is mentioned. With improved methods of illumination and of observation and better control of the state of adaptation, it has long become clear (Blaauw, 1914) that the response is graded and, in fact, very reproducible. Recently, Wassink and Bouman (1947), disregarding the later literature on the subject, have attempted to interpret some very early data of Blaauw (1909) on phototropism in Phycomyces in terms of an all-or-none phenomenon initiated by a one-quantum-per-cell process. We consider such an interpretation to be at variance with every fact concerning phototropism established since 1909.

period, the same adaptation curves, and the same action spectrum,[7] with a maximum in the blue and a cut-off at around 5,500Å (Castle 1931). Both responses are confined to the growing zone, both as to output and to input. The tropic response is intimately connected with the dioptric properties of the growing zone. The growing zone acts like a cylindrical lens which focuses laterally incident parallel light on a line a little outside the back surface. If this converging lens is converted into a diverging lens by immersing the sporangiophore in a medium of higher refractive index than that of the sporangiophore (mineral oil), then the positive phototropism is converted into a negative phototropism (Buder 1918, 1920). We infer that in the tropic response we are dealing with an altered distribution of growth *in space*, just as in the growth response we are dealing with an altered distribution of growth *in time*. Up to this point all theories connecting the tropic and the growth response agree with each other. They differ in details. Blaauw (1914) considered that the converging lens caused the back wall to be illuminated with higher intensity and invoked this fact to explain the tropic response. He argued simply that the back wall should give a greater growth response than the front wall. This argument is somewhat nebulous since the focusing obviously does not increase the total amount of light received by the back wall; it alters only its spatial distribution. Castle (1933) assumed that the absorbing pigment was not located in or near the wall, but distributed uniformly throughout the protoplasm. He pointed out that, due to refraction, the average length of path traversed by the light in the back half is about 20% greater than that traversed in the front half, and that therefore the net absorption in the back half might exceed that in the front half if, as is plausible, the attenuation of intensity during the traversal is slight. Buder (1946) argued that Castle's assumption of a uniform volume distribution of pigment was an unlikely one, and reverted to Blauuw's original approach. Conceding that the total amount of light striking the back wall cannot be larger than that striking the front wall, and in fact, must be somewhat smaller, he argued that the growth response in the back wall would be concentrated to a narrow zone around the

[7] Blaauw and van Heyningen (1925) report on a negative growth response of Phycomyces to exposure to ionizing radiations. We have attempted to verify these observations, with completely negative results.

Added in proof: A. G. Forssberg (*Acta Radiologica*, Supplement 49, 1943) has published a detailed study of the negative growth responses of Phycomyces to ionizing radiations. This important paper has only now come to our attention. According to Forssberg these growth responses disappear in the presence of very slight air currents. This may explain our failure to observe these responses.

midline, and that this should give the growth response in the back wall a *mechanical* advantage over the uniformly distributed one in the front wall.

Each of these three theories, as well as several other, less respectable ones, neglect an important fact about the tropic response, namely, that it occurs principally in a short section of the growing zone located near its base. This is a very obvious fact upon inspection, not accounted for by any of the theories. Indeed, if the tropic response were not confined to the base of the growing zone, but distributed uniformly along its length, spiral growth subsequent to the tropic response would cause the growing zone to *bend into a helix*. Consider, for instance, a level near the middle of the growing zone. If the sporangiophore above this level at a certain time had a bend to the right, then, due to spiral growth of the section of the growing zone *below* this level, this bend would be carried around into various directions quite unrelated to that of the incident light. It is therefore a necessity for the plant to confine its tropic response to the base of the growing zone. This obviously calls for a very special mechanism about which we have as yet no hint.

Prompted by these considerations, we were led to study the question whether perhaps the growth response, too, might be confined to the base of the growing zone. A direct approach to this question involving markers along the growing zone would be technically quite difficult. We attempted an indirect approach by confining the stimulation to short sections of the growing zone (.25 mm long). This was accomplished by a collar arrangement which shielded all but the desired section. Successive sections were tested with stimuli giving maximum response. The maximal responses to such stimulations were much smaller than those obtained with maximal stimulation of the entire growing zone: about one-eighth, corresponding to the ratio of the exposed length to the total length of the growing zone. Each section gave about the same response with the possible exception of the top-most one, where the response may be smaller. The shapes of the response curves, particularly those indicating their latent periods, were identical. These findings strongly suggest that the sections respond independently of each other, and that the response of the whole growing zone consists of a simple superposition of the responses of the various sections. It is, of course, conceivable that in each case the stimulations were conducted to the base and produced their response there, but in that case it would be difficult to understand why the latent periods should all be alike. However, a more direct test still seems desirable.

We have attempted to decide whether the changes in the level of adaptation produced by a stimulus are also confined to the exposed region. Our experiments suggest that this is true at least to first approximation, but we hope to explore this question with a more refined technique.

Another aspect of the tropic response concerns its dependence on the angle of incidence. If the specimen grows vertically and the light comes from above there is no tropic response. If it comes from the side, the tropic effect is presumably maximal. How does the effectiveness vary when we change the angle of the incident light with the axis of the sporangiophore between zero and 90°? We were led to go into this question by the following observations: for the study of the growth responses we used originally two opposing light sources giving incident light from the right and left. Under these conditions, a specimen growing vertically is in equilibrium when the two intensities are exactly equal. Random disturbances, however, do occur, causing slight deviations from vertical growth, and if they occur during measurements of the growth response, they cause gross errors in the measurements of the growth velocities. It is necessary to reduce these deviations from the vertical as much as possible. At first we did this by inserting glass plates in the light path of the right or the left source, imagining that if the light intensities are equal, the sporangiophore should return to the symmetric position, i.e. vertical growth. Eventually it was realized, however, that under these conditions vertical growth is not a *stable* equilibrium but an *indifferent* one. If the specimen is made to have initially any other direction relative to the line connecting the light sources, it is just as much in equilibrium in these positions. We may interpret this finding by an analysis involving a vector decomposition of the incident light into components parallel and at right angles to the axis of the specimen (Fig. 6A). In the case just considered of two horizontal beams coming in opposite directions and an axis of the specimen making an angle a with the vertical, the transverse components will cancel each other, whatever the angle a. The case is different when the two light sources make an angle of $180° - 2b$ with each other (Fig. 6B). Under these conditions the transverse components will cancel only if the angle of the specimen with the midline between the two sources is zero. In any other position there will be a net component in such a direction as to drive the specimen back to the symmetric position.

We assume that an illumination with the intensity S, coming from a

direction making the angle c with the axis of the specimen, exerts a turning moment on the specimen which is proportional to the transverse component of the illumination, or to $S \sin c$. The axis of this moment is at right angles to the plane containing the vector S and the axis of the specimen. Let us introduce a unit vector P in the direction of the axis of the specimen. We may then express our assumption by saying that the turning moment M is equal to the vector product $S \times P$. Let us assume further that the turning moments exerted by several light sources illuminating the specimen simultaneously add vectorially. To

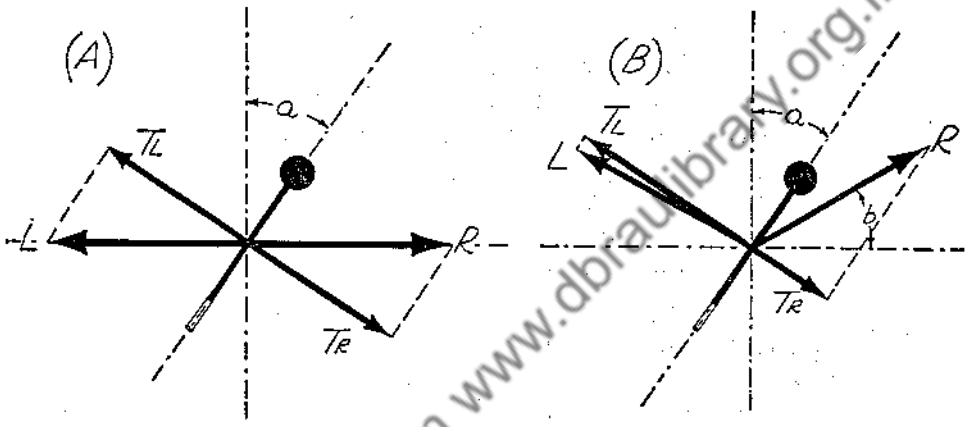


Fig. 6. A vector diagram illustrating the net phototropic effect of two equal light sources, L and R, on a specimen whose axis is deviating from the vertical by the angle a. In (A) the direction of illumination is horizontal. In (B) the direction of illumination makes the angle b with the horizontal. In (A) the transverse components of the illumination, T_L and T_R, cancel, whatever the direction of the specimen. In (B) the transverse components in general do not cancel. They cancel only if the axis of the specimen is vertical ($a = 0$).

obtain the resulting turning moment we may then add the various vectors S_i before forming the vector product with P. Thus in the case corresponding to our experimental arrangement (Fig. 6B), where the two illumination vectors lie in a vertical plane, symmetric around the vertical, each forming an angle b with the horizontal, and of equal intensity, the resultant illumination vector points vertically down and has the length $2S \sin b$. The turning moment exerted by this vector on a specimen deviating from the vertical by the angle a (in whatever direction) is equal to $2S \sin a \sin b$, and is directed so as to bring the specimen back to the vertical.

These predictions of the analysis are borne out by the test. Accordingly in our later experiments on growth responses we arranged the

light sources to give an angle of incidence of 30° above the horizontal (Fig. 1). This arrangement very effectively controls the drifting away of the specimen from the vertical. The control is still not ideal since the tropic response has a time delay of a few minutes resulting sometimes in slight oscillations about the equilibrium direction.

VI. THE RANGE ADJUSTMENT

So far we have not said anything specific about how big the stimuli have to be to produce these responses, i.e. about the sensitivity of our specimens. It was noted very early that the sensitivity depends enormously on the intensity to which the specimen had been adapted prior to the stimulus, or more generally speaking, on its past history with respect to illumination. Roughly speaking, the stimuli have to be increased proportionally to the adapting intensity, a relation analogous to the Weber law, valid over a range of four or five powers of 10. To be able to assess this relation quantitatively, we have to introduce suitable measures of the stimulus and of the reaction. For the stimulus it is obvious how to do this. Since there exists a reciprocity relation between time and intensity we introduce the product of time (in minutes) and intensity (in our units) as a measure of stimulus size and confine our stimuli to short ones, i.e. less than one minute. For the response, the procedure is not so obvious. Since the response is a graded one, we have to choose a particular response as a standard of comparison, and then measure the sensitivity in terms of the stimulus needed to produce this response. Early observers have chosen as this particular response the just perceptible response. This is a procedure which suggested itself by analogy with all-or-none systems: one measures whether or not there is a response and then calls the smallest stimulus which produces a response the threshold stimulus. This procedure is quite impractical for two reasons: in the first place, as we have seen, the threshold is not a characteristic quantity of the specimen, but of the resolving power of the measuring technique. In the second place, since it means operating near the limit of the resolving power of the technique, this procedure introduces exceptionally large errors. For these reasons we have chosen to use as a standard response not the smallest measurable one, but an intermediate one, located in a region in which the measurable response changes most rapidly with the stimulus and is readily measurable. From what we have said above about the general characteristics of the response as a function of stimulus, it is clear that we do not need to meas-

ure the entire response curve each time in order to get a measure of the intensity of the response. We have chosen as a measure of the response the ratio of the growth during the period from 2.5 to 5 minutes after the stimulus to that during the period from 0 to 2.5 minutes. The period from 2.5 to 5 minutes takes in most of the positive phase of the response, and the period from 0 to 2.5 minutes gives the base line of normal growth. This ratio will be designated as R. Fig. 7 shows how R

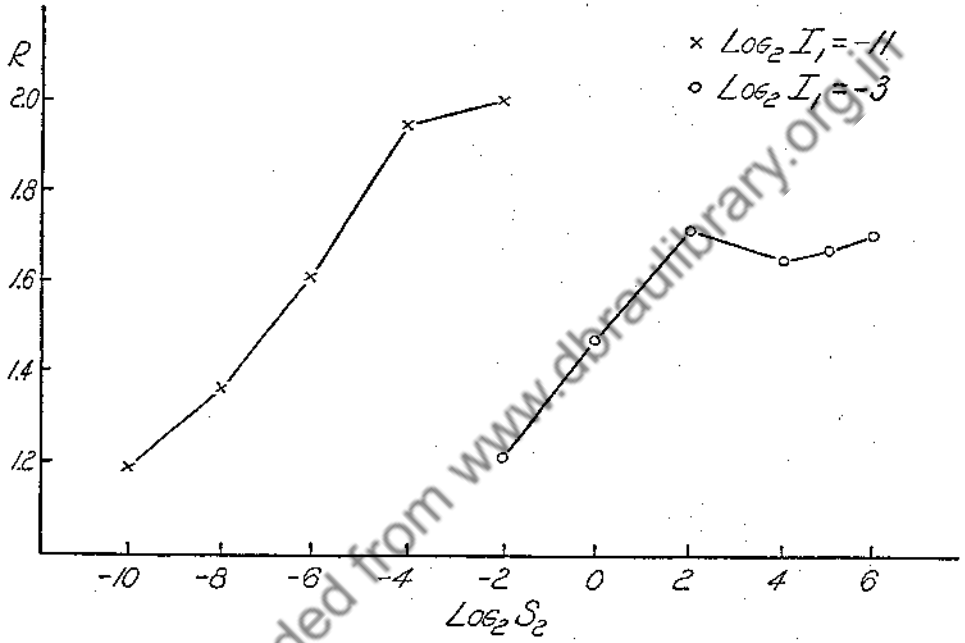


Fig. 7. The response R versus the logarithm of the stimulus S, for specimens previously equilibrated either with $I_1 = 2^{-11}$ or with $I_1 = 2^{-3}$. Each response measured four times and averaged. Stimulations given at 20 min intervals. All measurements referring to equilibration with a given intensity were taken on a single specimen. The smaller maximum response in the case of $I_1 = 2^{-3}$ as compared to $I_1 = 2^{-11}$ was a characteristic of the specimen used. It is not a characteristic of the level of adaptation.

varies as a function of log S, for two different adapting intensities I. R varies from 1 for no response to about 2 for maximum response. The curves are sigmoid, and nearly straight for responses between about 1.2 and 1.8. There is a good deal of variation between replicate measurements taken on the same specimen. However, the averages of such replicate measurements are very nicely reproducible from one specimen to another. A single specimen has a useful life of about 10 hours, and for measurements of this type a measurement can be made every 20

minutes.[8] The two adapting intensities employed in the experiment represented in Fig. 7 differed by a factor 256, or eight log 2 units. It will be seen that the corresponding R (log S) curves are quite similar and are displaced by eight log 2 units relative to each other. Here, then, we have the quantitative expression of the Weber relation. We note, further, that the R (log S) curve is steepest for about $R = 1.4$ and that our specimens give this response when log $(S/I) = 3$ or $S/I = 8$ minutes. In other words, the amount of light given in a short stimulus must be equal to that given by 8 minutes of adapting illumination in order to produce a response $R = 1.4$. Finally, we note from Fig. 7 that under the conditions of this test the response is, in fact, exceedingly graded. An increase in response from the smallest conveniently measurable one (about $R = 1.2$), to maximum size requires an increase in stimulus of about five log 2 units, or a factor 32. The measurements reported in this paper are confined to the region of perfect range adjustments. Our measurements outside this range, near the limit of dark adaptation and under light saturation, are still incomplete. We hope to report about these at a later date.

VII. THE LEVEL OF ADAPTATION

The preceding experiments tested the sensitivity of the specimens after they had been brought to equilibrium with an adapting intensity of illumination. This equilibration resulted in an adjustment of the state of the specimen in such a manner that the effect of the subsequent stimulus depended on the ratio S_2/I_1, where S_2 is the test stimulus and I_1 is the conditioning intensity. We wish to introduce a measure for the level of adaptation, i.e. we want to introduce a quantity which in some manner characterizes the sensitivity of the specimen at any given moment, whether it is in equilibrium or not. Evidently our measure of the level of adaptation will be most accurate if it utilizes responses in a

[8] There are numerous reports in the literature to the effect that each growth response is followed by a refractory period of 20-30 minutes. This concept can be defined in a meaningful manner only for threshold systems. For a graded system it is to be replaced by stating the increase in the level of adaptation produced by the stimulus. Oort (1932) was the first to show clearly that the stimulus which produces the response also raises the level of adaptation so that a repetition of the same stimulus after a short interval need not produce the same response. When the specimen is highly dark-adapted before the first stimulus the response to the first stimulus may be much larger than the response to a second stimulus given a few minutes later. If the technique of measurement is crude the first response may be picked up but not the second, thus simulating a refractory period. The anomalously large increase in A produced by very large stimuli enhances this effect. Oort showed that stimuli only five minutes apart produce very distinct responses.

region where the response changes most rapidly with the stimulus. A response $R = 1.4$ seems a suitable choice for this purpose. Let us agree, therefore, that we determine the level of adaptation by finding that stimulus size which gives a response $R = 1.4$. To do this we need not stimulate the specimen with exactly this critical stimulus, but may use smaller and larger stimuli, measure the response for each, plot these responses versus the stimulus, and interpolate the critical stimulus from such a graph. In the preceding section we pointed out that after equilibration with the intensity I_1 the critical stimulus size is $S_2 = 8I_1$. Our measure of the level of adaptation should obviously be proportional to the critical stimulus. We could make the proportionality factor equal to 1, but this would be somewhat arbitrary since the critical stimulus was defined with the aid of an arbitrarily chosen standard response. We arrive at a more rational choice by the following line of thought. We ask ourselves: with which intensity would we have to equilibrate the specimen in order to bring it to the same level of adaptation? This we will call the *equivalent intensity*. It may be obtained by dividing the critical stimulus by eight minutes. We will give this intensity the name A. This definition of the level of adaptation is independent of the particular choice of the standard response. After equilibration with I_1, the level of adaptation is by the definition $A = I_1$. We are now in a position to outline a procedure for determining A also for some non-equilibrium states. The procedure consists in bringing the specimen into the particular state, testing it with various stimuli, determining by interpolation the stimulus giving response $R = 1.4$ and dividing this stimulus by 8. Fig. 8c gives an example of such a determination of A. The principal and unavoidable limitation of this procedure is that the conditioning program itself may give a response which interferes with the measurement of the response to the test stimulus. This interference is particularly large if the conditioning program consists of a strong, short stimulus. In that case we have to wait until the response to the conditioning stimulus has run its course before we can determine A, i.e. we have to wait about ten minutes before A can be determined. The interval from 0 to 10 minutes after a strong stimulus is thus inaccessible to direct determinations of A. We may only hope to infer the earlier values of A by the somewhat dubious procedure of extrapolation backwards from measurements of A at later times. A similar, but less stringent limitation is present in the case where the conditioning program consists in a long period of illumination, followed by a step-down to zero or to some other intensity at time zero. In this case, too, the condition-

ing program has an output of its own, in the interval between 0 and 10 minutes after the step-down. Here, however, we are on safer ground for determining the kinetics of A in the inaccessible interval since its value at time zero is known from equilibrium measurements to be equal to I_1.

Before proceeding with the presentation of our experimental data, let us briefly restate the immediate goal. Our observational data concern growth velocities in response to certain illumination programs. We will speak of the *growth output* in response to an *illumination input*. We have seen that the functional relation between these two is enormously influenced by a variable describing the internal state of the specimen, which we have called the level of adaptation, A, and for the measurement of which we have outlined a procedure. The physical nature of this variable will be left open entirely. We only know that the variable itself is determined by the illumination program, and it constitutes, therefore, another and perhaps more immediate output of the illumination input. We will refer to it as the *adaptation output*, and our immediate concern is a description of the functional relations between the *illumination input* and the *adaptation output*. To explore this relation we measure A as a function of time in response to either a short (15 sec) stimulus of various sizes, or after equilibration (30 min) with various intensities. Figs. 8a and b show the results of such measurements. In each case A appears to drop at first exponentially, by a factor 2 in 2.5 minutes after the light is turned off. For longer times A drops more slowly. The curves were not followed beyond the 30 minute point for the following reason: the average growth rate of the specimens is 3 mm per hour. The length of the growing zone is about 2 mm. In 30 minutes, therefore, a large part (about half) of what was the growing zone at time zero has become secondary wall and has been substituted by the stretching of what was the upper part of the growing zone at time zero. This replacement will surely complicate the interpretation of the measurements and these longer periods should therefore be left out of consideration when attempting to analyze the principal mechanism responsible for the adaptation output.

When comparing the changes in A after equilibrating with various intensities (Fig. 8a) we find that the curves run parallel except for the lowest one which is too close to maximum dark adaptation. The curves are displaced relative to each other strictly in proportion to the equilibrating intensity.

The curves referring to a conditioning program consisting of pre-

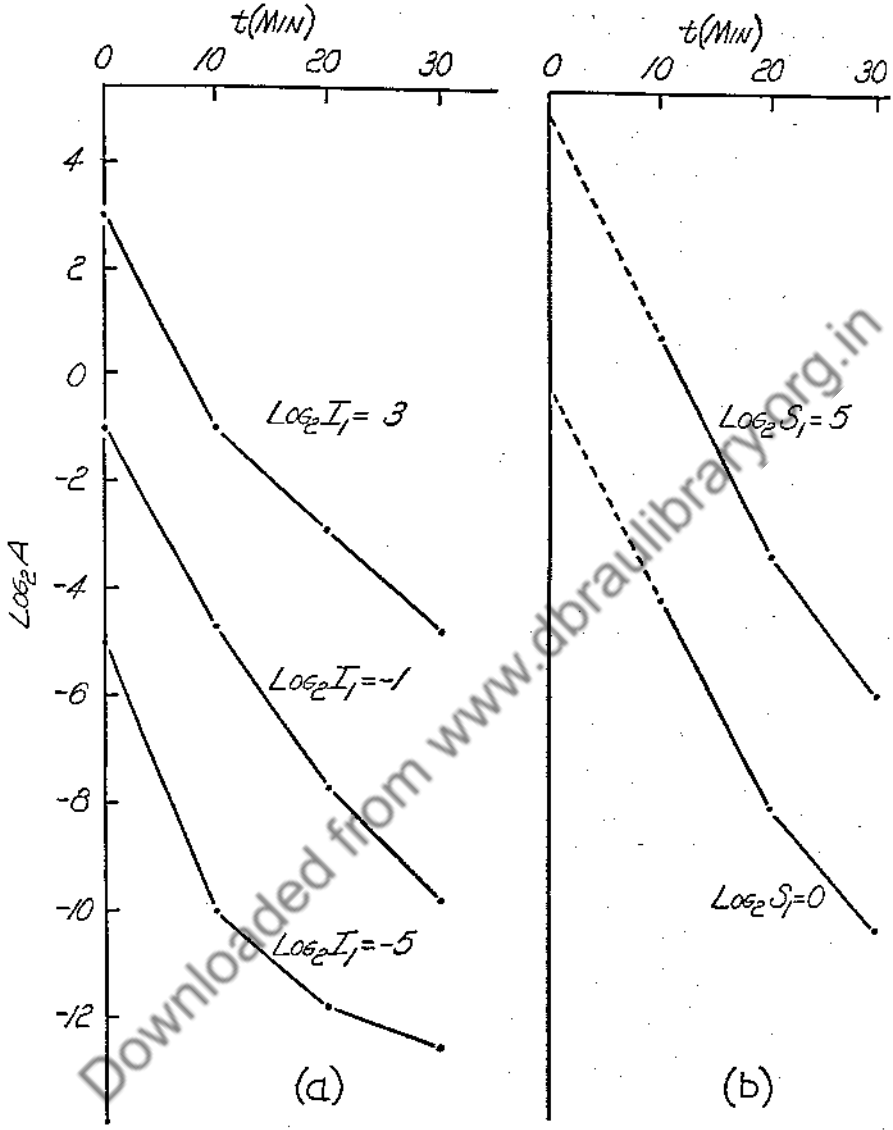


Fig. 8. The logarithm of the level of adaptation versus time in the dark, (a) after previous exposure for 30 min or, (b) for .25 min to various intensities I_1. In the case of the short conditioning exposures the product $S_1 = I \times t_1$ is indicated in the graph. Each level of adaptation was determined from a set of measurements as illustrated in Fig. 8c.

equilibration to a very low intensity followed by a short strong stimulus (Fig. 8b) show the following characteristics: The drop in A between the 10 minute and 20 minute point is as fast as after equilibrating with a constant intensity. This suggests that also during the first ten minutes

where the changes in A have to be inferred by extrapolation, they are similar in the two cases. Performing the extrapolation, we arrive at a value of A immediately after the stimulus. Concerning this value we note, in the first place, that it is proportional to the size of the conditioning stimulus, suggesting that A increases linearly during the

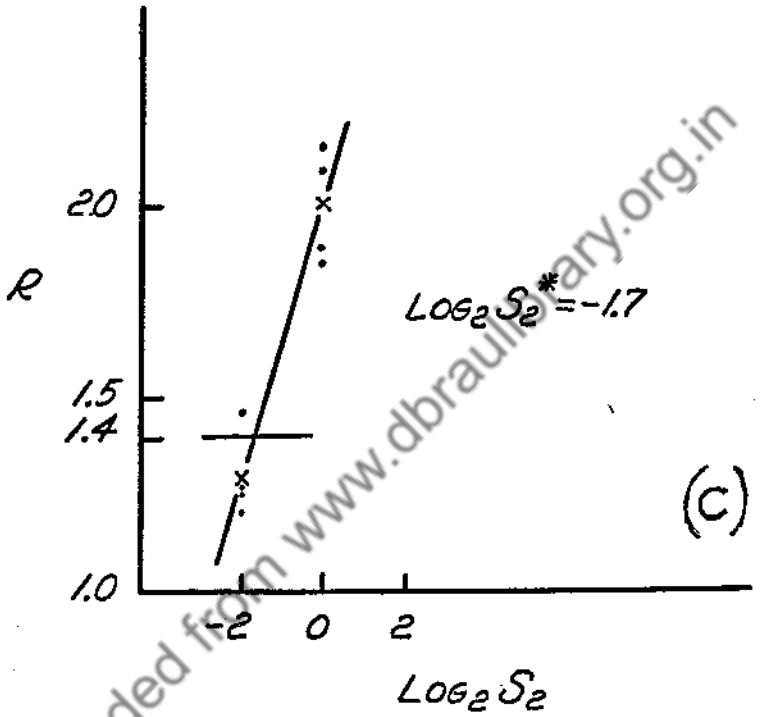


Fig. 8c. Response versus stimulus for a specimen dark adapting for 10 min after previous equilibration for 30 min with $I_1 = 2^{-1}$. Equilibration was restarted 10 min after each test stimulus. Thus one cycle consisted of $30 + 10 + 10 = 50$ min. Test stimuli $S = 2^0$ and 2^{-2} were used. Each stimulus was tested four times. The dots represent individual measurements, the crosses the averages. The line connecting the averages intersects the standard response $R = 1.4$ at the critical test stimulus $S^* = 2^{-1.7}$. From this we obtain $A = 2^{-1.7}/8 = 2^{-4.7}$. The difference in slope between this line and those in Fig. 7 is spurious. Our experiments have not revealed any systematic trend in this slope.

short stimulus, at a rate proportional to the intensity. In the second place, our results enable us to evaluate the absolute value of this proportionality constant. Consider, for instance, the upper curve, where the conditioning stimulus consisted of an illumination with the intensity $I = 2^7$ for 15 sec. The extrapolated value for A immediately after the stimulus is 2^5. We know that if the illumination had not been a short one, but continued indefinitely A would have reached the value 2^7,

or 4 times higher than that reached in 15 sec. In other words A, rising at first linearly, reaches ¼ of its final height in 15 sec. Its time constant, therefore, is 1 minute. This time constant refers to the *initial* rise in A when the specimen is illuminated with an intensity high compared to its level of adaptation. This time constant is considerably shorter than the time constant measured for the decay of A, 3.8 minutes.

This sharp rise in A which occurs when the specimens are subjected to a very strong stimulus (relative to the level of adaptation) is difficult to fit into any simple scheme describing the kinetics of adaptation. We suspected that it represented an anomaly, characteristic only of very large stimuli, and decided to test the adaptation output in response to smaller stimuli. The conditioning program consisted of a 30-minute exposure to $I_0 = 2^{-1}$ followed immediately by a stimulus $S_1 = 2^2$, followed by darkness. The level of adaptation was tested at 10 minutes after S_1 and was found to be equal to $A(10) = 2^{-3.6}$. Extrapolated back to time zero, this gives $A(0) = 2^{0.4}$ (The conditioning stimuli in this experiment were given in half the trials with the intensity 2^4, duration 15 sec, and in the other half of the trials, with the intensity 2^6, duration 3.75 sec. These two conditioning stimuli resulted in the same level of adaptation, showing that the reciprocity relation between time and intensity holds not only for the growth output, but also for the adaptation output, in the range tested.) The change in A produced by the stimulus (from 2^{-1} to $2^{0.4}$) permits us to calculate the time constant for the rise in A, and for this experiment we find the value 4.9 minutes, close to the time constant of the decay of A in the dark.

These findings, meager as they are, encourage us to conjecture the general functional relation between $I(t)$ and $A(t)$, valid for relatively small variations in A. They are described by the differential equation

$$dA/dt = (I - A)/b \qquad (1a)$$

where b is the time constant of the system, approximately equal to 3.8 minutes. This equation obviously satisfies the two basic findings, viz., that $I = A$ after equilibration with a constant intensity, and that A decreases in the dark exponentially with the time constant b, irrespective of its initial level, and irrespective of how it was brought to this level.

For an arbitrary illumination program $I(t)$ this equation has the integral

$$A(t) = A(0)e^{-t/b} + (1/b)\int_0^t I(t')e^{(t-t')/b}dt' \qquad (1b)$$

Equation 1b implies that during a short stimulus A increases by $(1/b) \int I(t')dt' = S/b$. Designating the values of A before and after the stimulus as A_- and A_+ we therefore have the relation

$$A_+ = A_- + S/b \tag{1c}$$

The functional relation between input and adaptation output implied in equations 1a and 1b is a linear one, and one may hope that the true relation can be approximated by it, at least within limits still to be explored.

VIII. THE COUPLING BETWEEN ILLUMINATION, ADAPTATION, AND GROWTH OUTPUT

The relation between $I(t)$ and $A(t)$ discussed in the preceding paragraphs is by itself hardly verifiable, because the definition of A involves the measurement of growth responses to test stimuli *undisturbed by growth outputs of the conditioning program*, and this restriction very severely limits the possibilities for direct tests. We must therefore attempt to free ourselves of this restriction by adding another conjecture regarding the manner in which different growth outputs do or do not interfere with each other. Here it is clear in the first place that there exists a level of saturation for the growth output and that we cannot expect to find a simple situation unless we stay below this level. Secondly, from the fact that stimuli and adaptive levels have to be raised proportionally to produce the same growth output, we infer that what is relevant for the growth output is the ratio of I to A. This ratio we will call the *subjective intensity* and call it

$$i = I/A \tag{2}$$

Let us consider what happens to the subjective intensity during and after a short stimulus S_1 superimposed upon a constant background intensity, I_0. During a short square-shaped stimulus of intensity I_1 and duration t_1, $I_1 \times t_1 = S_1$, A increases according to equation 1b linearly from $A_- (= I_0)$ to $A_+ = A_- + S_1/b$. Therefore, during the stimulus we have the relation

$$A(t) = A_- + St/b\ t_1 \tag{3}$$

Since this increase of A during the stimulus may represent an increase by a large factor, the *subjective* intensity, jumping from unity to a high value at the beginning of a stimulus, may decrease by a large factor even during the shortest stimulus. This decrease has to be taken into

account in an evaluation of the spike transient in the subjective intensity, which we conceive to be the quantity immediately responsible for the growth output. Let us define the *subjective stimulus* s as the integral of this transient during the stimulus. In the present case this works out as follows:

$$s = \int_0^{t_1} I_1/A \, dt = \int_0^{t_1} I_1/(A_- + St/bt_1) \, dt = b \log(1 + S/bA_-) \quad (4)$$

For small subjective stimuli, i.e. for stimuli which are small compared to those which give the standard response, the logarithm may be developed into a power series of S/bA_- and the subjective stimulus becomes equal to the first term of this series. We then have $s = S/A_-$ and s is proportional to S. For larger stimuli, however, s increases only as the logarithm of this ratio.

Before we proceed let us point out and resolve an apparent paradox in this relation between the subjective and the objective stimulus. As we have seen, this relation is not a proportional one, except for very small stimuli (i.e. doubling of the objective stimulus does not double the subjective stimulus), and yet we have a reciprocity rule between time and intensity of the stimulus. If we double the duration of a stimulus, we get the same effect as by doubling the intensity of the stimulus. In the first case mentioned, when we double the duration, the subjective stimulus increases less than proportionally because the second half of the stimulus finds the specimen at a higher adaptive level than the first one. Conversely, if we double the intensity of the stimulus, the rate of rise of the level of adaptation is increased, so that also in this case the subjective stimulus increases less than the objective stimulus. Our formal integration demonstrates that, in fact, in both cases the end result is the same. In other words, it shows that the subjective stimulus is a function only of the objective stimulus and of the initial level of adaptation, and does not depend on the shape of the stimulus, as long as its duration is short compared to the time constant of the system.

The subjective intensity i has another important property. For an arbitrary illumination program which is preceded and followed by equilibration with the same intensity I_0, we have the identity

$$\int (i - 1) \, dt = 0 \quad (5)$$

(This is easily proved by substituting I/A for i, and then $A + b \, dA/dt$ for I, and observing that $A = I_0$ asymptotically both for large negative and positive times.) The relation means that the positive and negative

deviations of i resulting from any program which returns to the original intensity cancel exactly.

If the program goes from equilibration with I_0 through any intermediate course to equilibration with another intensity I_1, then we have the relation

$$\int (i - 1)\, dt = b \log (I_1/I_0) \tag{6}$$

Both these relations are of interest in connection with our attempt to establish a relation between the subjective intensity and the growth output. For the growth output we know that its net value is zero if the program consists of a short stimulus and that it is positive or negative for a step-up or a step-down program, respectively.

A similar correlation may be noted between the subjective stimulus and the growth output in their dependence on the size of the objective stimulus. For intermediate stimulus sizes both are proportional to the logarithm of the ratio between the objective stimulus and the adapting intensity. These relations suggest that the *subjective intensity* may be the important variable which quite generally stands in a linear functional relationship to the growth output. By this we mean the following: at equilibrium the subjective intensity is always unity. Under the influence of a given illumination program it will deviate from unity in a predictable manner. Let us assume that we could design illumination programs resulting in a subjective intensity which deviates from unity only during a short period. This we will call a subjective pulse. Such a subjective pulse will lead to a growth output represented by a certain function of time. We now postulate two things: first, that the growth output for subjective pulses of various sizes is equal to the output produced by a unit subjective pulse multiplied by the actual size of the pulse, and secondly, that the growth output for an arbitrary illumination program can be calculated as a simple superposition of the outputs of all the pulses into which the subjective intensity can be decomposed. These two postulates are formulated analytically in equation 7, in which $Dv_1\ (t)$ represents the growth output due to a unit subjective pulse, $Dv(t)$ represents the actual output resulting from an arbitrary illumination program, and $Di(t)$ represents the subjective intensity output of this program. The letter D expresses that we are referring to deviations from the equilibrium values of the velocity and of the subjective intensity, respectively.

$$Dv(t) = \int_{-\infty}^{0} Di(t - t')\, Dv_1\ (t')\, dt' \tag{7}$$

Let us test the validity of these postulates at first qualitatively by comparing theoretical predictions and the experimental findings in the growth outputs of the four basic illumination programs: pulse-up, step-up, pulse-down, and step-down.

The growth outputs were shown in Fig. 3. The theoretical predictions for the level of adaptation and the subjective intensity are given in the upper half of the same figure.

Comparison of the growth outputs with the subjective intensities shows the following similarities:

1. Step-up. The i output jumps to a high value at the moment of the step and then falls back rapidly to its equilibrium value. The growth output shows a positive phase only, declining more slowly than in the case of a short objective stimulus.

2. Step-down. The i output drops to near zero at the moment of the step and then returns to its equilibrium value, but only very slowly. The growth output shows a negative phase only which is shallow and extends longer than in the case of the step-up. The onset of the response cannot be determined reliably since the amplitude is small, but the minimum is clearly reached later than the maximum in the case of the step-up.

3. Pulse-up. The i output consists of a sharp positive spike followed by a shallow negative variation. The growth output starts out like the output for the step-up, but the slow declining phase of the step-up is replaced by a shallow negative variation. The net output, both of i and of v is equal to zero (see equation 5).

4. Pulse-down. The i output consists of a very small negative spike followed by a small and shallow compensating (see equation 5) positive variation. The growth output is correspondingly small and seems to consist also of a small negative variation followed by a compensating positive variation.

We have attempted to test the linear functional relationship between $i(t)$ and $v(t)$ in another manner which is less direct but more accurate, involving medium size periodic stimulations superimposed upon a constant intensity, and comparing the growth outputs for two such programs in which the periods differ by a factor two. The programs, the i outputs (calculated), and the v outputs (observed) are illustrated in Fig. 9. We cannot predict the v output for either one of these programs until we know the basic response function $v_1(t)$. However, it can be shown very easily that the v outputs of the two programs should be related to each other by the following equation:

$$Dv_T(t) = Dv_{2T}(t) + Dv_{2T}(t + T) \qquad (8)$$

where T is the shorter of the two periods. This equation may be expressed by saying that the v output of the T program is obtained from the v output of the $2T$ program by superimposing the first and second half of the latter over each other. The circles in the growth output of the T program were calculated in this manner from the growth output of the $2T$ program. They show very good agreement with the experimental curve. Actually the application of equation (8) presupposes that the specimens come to adaptive equilibrium with I_0 in the intervals between stimulations. In our experiments, this condition was met well in the $2T$ program, but somewhat imperfectly in the T program. As a result, the subjective stimuli were probably a little smaller in the T program than in the $2T$ program. The v outputs should be proportional to these subjective stimuli. The size of this correction depends on the precise value of the time constant b of dark adaptation. If we take for this constant the value estimated from our dark adaptation experiments, i.e., $b = 3.8$ minutes, the correction amounts to about 25%. This seems rather more than our experimental error, and suggests that we may have underestimated b. One may hope that a refinement of this experiment may be useful for determining the precise value of b.

IX. DISCUSSION

The theoretical field of biological range adjustment may be said to consist of a large void occupied by a sole highly moribund inhabitant. This inhabitant is the theory proposed by Selig Hecht in 1919. It refers specifically to sensory systems in which the quality of the stimulus is light and it involves the basic assumption that the sensitivity is directly related to the concentration of the light absorbing pigment. It assumes that the illumination bleaches the pigment, and that the pigment concentration present at any one time is determined by the balance of the bleaching reaction and certain dark reactions which cause the pigment to be resynthesized. This theory was set up at a time when physical chemistry was beginning to make its entrance into biological theories, and full credit should be given to Hecht for the bold application of the principles of physical chemistry at a time when exceedingly little was known of the phenomena to be interpreted. From our present point of vantage, several exceedingly weak points in this theory are apparent. The first weak point concerns the fact that range adjustment is not confined to light sensitive systems, but is a very general characteristic of almost any sensory system. While it is easy to visualize how illumination in competition with dark reactions might shift the concentration

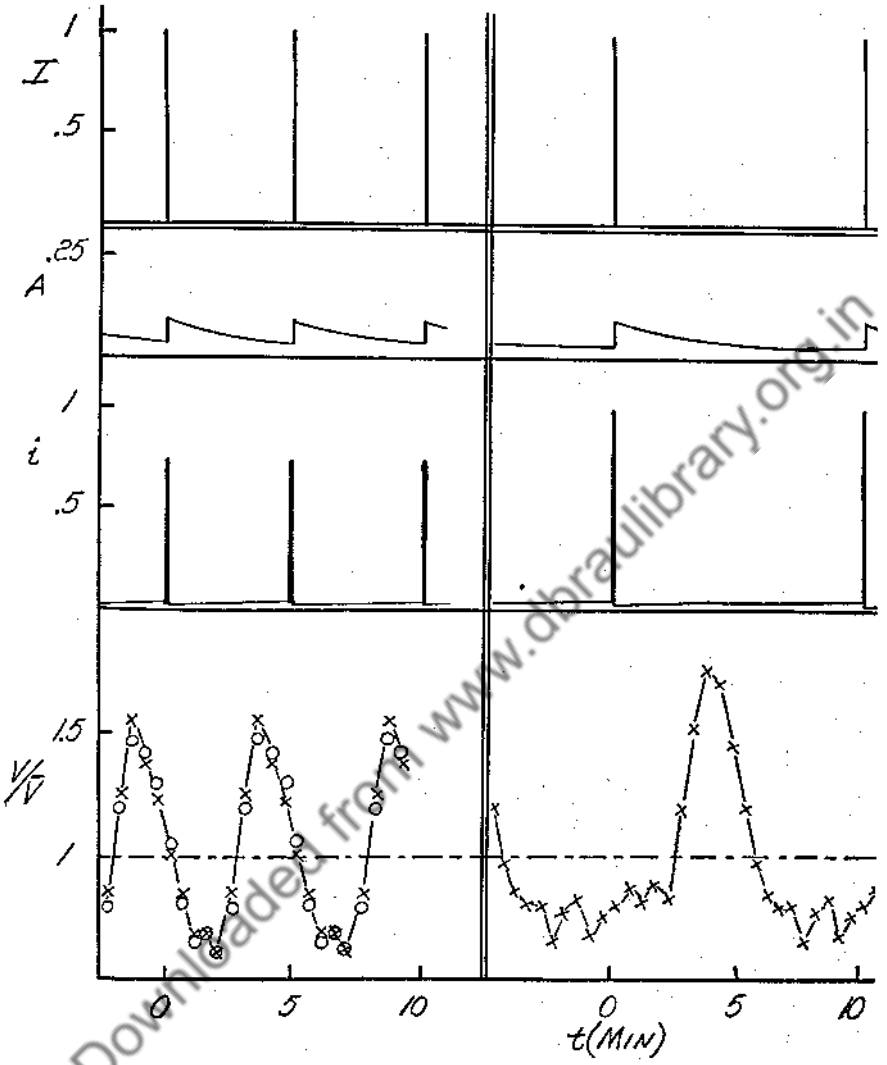


Fig. 9. Superposition of growth outputs. A specimen is subjected to two periodic stimulation programs (top row), with period $T = 5$ min (left) and $2T = 10$ min (right). In both programs $I_1 = 2^{-5}$ and $S_2 = 2^{-2}$. Duration of stimuli .25 min. The second and third rows show the calculated values of $A(t)$ and $i(t)$. The fourth row shows the growth responses averaged over five cycles in each case (crosses). The circles in the left diagram are obtained by superimposing the two halves of the output of the $2T$ cycle, as explained in the text.

of the primary receptor substance, there are no obvious analogous physical-chemical systems that could be set up in the case of such stimulus qualities as pressure or tension, etc. A second weak point of Hecht's approach is a quantitative one which has become very obvious only

during recent years. In the case of vision in vertebrates, for instance, the amount of bleaching caused by various conditioning intensities can be measured. It turns out that conditioning intensities which lower the sensitivity by a factor 100, and which therefore should have bleached 99% of the pigment, cause only a negligible amount of bleaching—a fraction of a percent (Hagins and Rushton, 1953). In this situation several attempts have been made to rescue the general notion of a relation between the concentration of unbleached pigment and the sensitivity. It might be thought that only a small fraction of the retinal pigment is functional. This is untenable because it is known that the quantum yield of bleaching for all of the pigment is near unity, and that in the dark adapted eye every absorbed quantum is functional. It has also been thought that the changes in sensitivity are not located in the sensory elements themselves, but at higher stations. It is known that several hundred of these primary elements (the rods of the retina) are tied together in some kind of coincidence-counting network which sums the outputs of the elements. One might think that the changes in sensitivity involve changes in the sizes of these groups of elements which are tied together. This is contradicted by direct evidence obtained by Rushton and Cohen (1954) who showed that the course of dark adaptation is not materially influenced by the size of the group of receptors put to the test. Of course, if this interpretation had been valid, it would have been the final blow to any attempts to establish a simple connection between pigment concentration and sensitivity. A curious attempt at rescuing the basic notion of Hecht's theory has recently been made by Wald (1954). Wald assumes that the 20-odd million pigment molecules contained in one sensory element are organized into some thousand separate compartments. For this idea there is very suggestive direct morphological evidence obtained in electron microscope studies. Wald assumes further that each compartment is a separately excitable unit, that it can be excited when a single pigment molecule within a compartment absorbs a quantum of light, and that the compartment is excitable only *if none of its pigment molecules are bleached.* It is obvious that this set of assumptions helps us out of the principal dilemma. With only a fraction of a per cent bleaching it gives us enormous changes in sensitivity if the bleaching of a single pigment molecule in a compartment containing, say, 20,000 pigment molecules makes this compartment inoperative. We believe, however, that a closer examination of the quantitative aspects of Wald's theory reveals a fatal weakness. Consider an intensity of illumination which raises the thresh-

old by a factor e^n. On Wald's notions this means that a fraction e^{-n} of the compartments are operative. Since the number of bleached pigment molecules per compartment should be distributed in a Poisson manner, this would mean that we have on the average n bleached pigment molecules per compartment. On any reasonable assumption about the kinetics of bleaching and resynthesis, in the steady state the average number of bleached molecules per compartment should go up proportionally with the intensity, at least in the region of very slight bleaching with which we are here concerned. This should mean then that when we increase the intensity of the conditioning illumination, the sensitivity should decrease exponentially, in flat contradiction with the facts. Similar arguments can be developed regarding the implications of Wald's theory with respect to the kinetics of dark adaptation, and these, too, lead to flat contradictions with the known kinetics of dark adaptation. We conclude, then, that there is no basis on which the notion of Hecht's can be supported, and that the mechanism of range adjustment must involve other stages of the system rather than the bleaching of the pigment.

A very similar argument for ruling out any theory involving bleaching of the pigment as a cause for the changes in the level of adaptation can be used in the case of Phycomyces. This argument runs as follows: although we do not know the precise nature of the pigment we can be certain that its molar extinction coefficient is not appreciably higher than that of other strongly absorbing pigments like visual purple, carotine, riboflavin, etc., possessing an absorption band extending over several hundred Å. Let us take visual purple as an example. Its extinction coefficient expressed as a cross section per molecule is equal to $q = .4 \times 10^{-16}$ cm². On the other hand, the intensities to which our specimens adapt rapidly correspond to a quantum flux of about $n = 2.5 \times 10^{10}$/cm² sec, or higher. At these intensities, then, any given pigment molecule will absorb a quantum once in every $1/nq = 10^6$ sec. Therefore, even if the pigment were bleached with a quantum yield of unity it would take of the order of 10 days before an appreciable portion of the pigment were bleached, a time scale several orders of 10 larger than the actual time scale.

Hartline and McDonald (1947) have studied the sensitivity changes of a single photoreceptor element in the eye of *Limulus*. The procedure here consists in mechanically isolating from the optic nerve a group of fibers which respond as a single element when the whole eye is illuminated. The criterion for "singleness" is a very obvious feature: a per-

fect, simple regularity in the sequence of discharges given in response to a brief stimulus. It is possible that this regularity means that we are dealing with the discharges in a single fiber, but this has not been proved. On the input side we are certainly dealing with a curiously complex system (Waterman and Wiersma, 1954). A single ommatidium contains about a dozen pigmented cells arranged symmetrically around the axis and lower down a single eccentric cell which is not pigmented. Fibers extend both from the pigmented cells and from the eccentric cell into the optic nerve. The fiber from the eccentric cell is thicker than the others and appears to be the only one which conducts impulses. The function of the thin fibers leading off from the pigmented cells is obscure. It is likely that the retinal potential is principally an expression of potential differences between different portions of the eccentric cell, generated by the light stimulus. Presumably the pigmented cells are affected by the light in the first instance. Secondarily the eccentric cell responds in a graded manner.[9] In the third stage this graded response of the eccentric cell causes a shorter or longer series of all-or-none responses in the fiber leading off from the cell. It is the pattern of responses recorded from the third stage of the system which may be used to assess the sensitivity changes of the whole system.

Hartline and McDonald (1947) have shown that there exists a characteristic relation between the number of impulses and the size of a test stimulus. This characteristic curve, if measured after equilibrating the system with a given conditioning intensity, is displaced to the right or left in proportion to the conditioning intensity (Fig. 3). Thus their data permit one to introduce as a measure of the level of adaptation, just as in our case, the "equivalent intensity," i.e. the intensity, equilibration with which gives the same relation between the number of impulses and the stimulus size as does the particular program under consideration. Introducing this measure and evaluating the data

[9] Mueller (1954) has attempted to interpret the available data on excitation in single elements of the eye of Limulus in terms of a theory involving the idea that only a small number of molecules are brought into an excited state by the stimulus. It is then postulated that every transition of an excited molecule A* into a state A' may initiate an impulse in the optic fiber. In this theory the primary response of the sensory cell is not a continuously graded one but is described by a small integer, which is subject to statistical fluctuations like a Poisson variable. Similarly, the output of impulses in the optic fiber resulting from a stimulus is subject to statistical fluctuations both as to number and as to the time sequence. The author calculates certain time *averages* and number *averages* and these show good agreement with the observed data. However, when a comparison is made between the predicted and observed *fluctuations* of these quantities, it is found that the observed fluctuations are much smaller than the predicted ones. This rules out the basic premise of the theory.

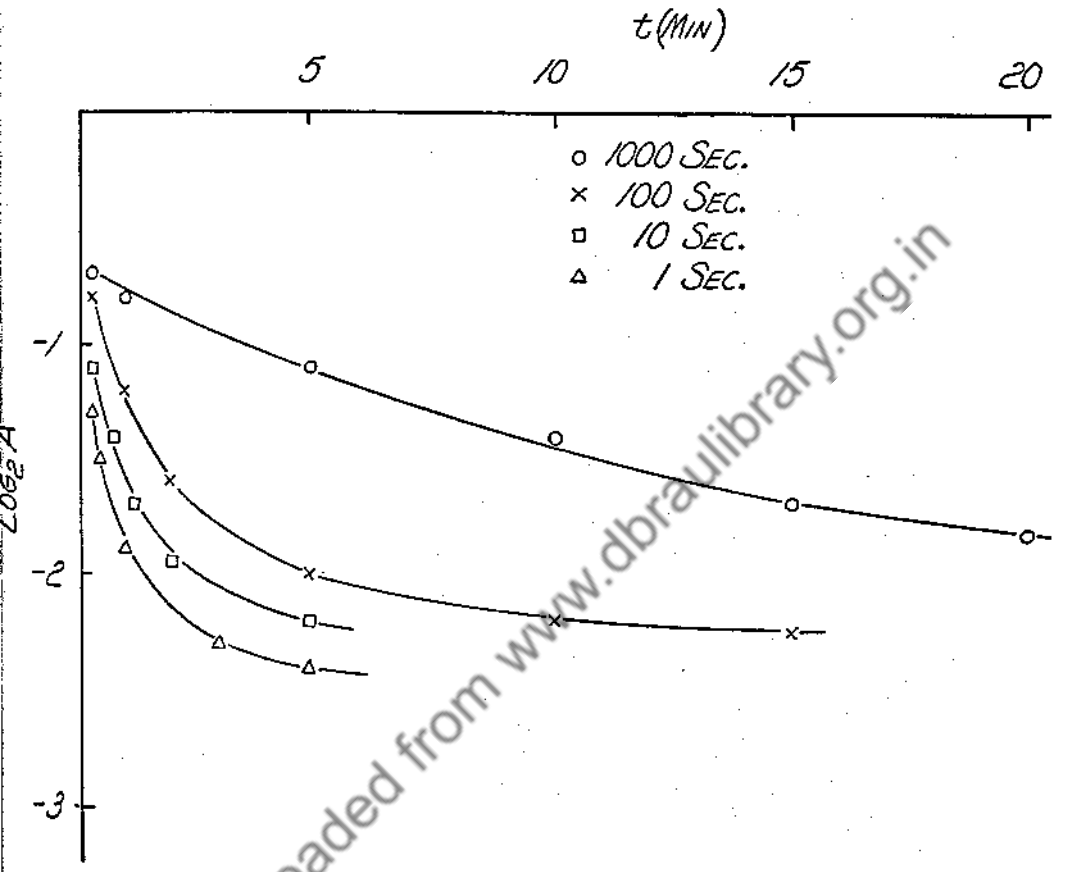


Fig. 10. The level of adaptation versus time in the dark after previous exposure for various durations to a constant intensity. Data for Limulus (Hartline and McDonald, 1947), evaluated as indicated in the text.

given by the authors in Fig. 4 of their paper we obtain the following dark adaptation curves subsequent to a series of conditioning programs involving a constant intensity and periods of exposure ranging from 1 to 1,000 seconds (Fig. 10). The principal point of interest is the great difference in the rate of dark adaptation following these different programs. The longer exposures lead to higher initial levels and slower

rates of dark adaptation. The principal factor controlling the rate of dark adaptation does not appear to be the duration of the exposure as such, but the initial level of adaptation attained during the conditioning program. This initial level is a function of the product intensity × time of the conditioning program (Fig. 6), over the impressively wide range of time from 1 to 100 seconds.

It is clear from these results that in Limulus the level of adaptation is not the only variable characterizing the internal state of this system, and this appears to be borne out by a lack of homology with respect to details of the pattern of discharges obtained after different conditioning programs. Such differences, the authors state in their 1947 publication, exist, but details have not yet been made available.

Our experiments have led us to the construction of a relatively simple picture for the light growth responses of Phycomyces. In the first place the connection between input and output appears to be strictly local. Each section of the growing zone responds according to the illumination program it receives, irrespective of the illumination of sections located above or below it, and the time course of these responses appears to be the same at each level. There are thus no complications from conduction phenomena, at least in the longitudinal direction, and the responses measured on the whole specimen represent a simple sum of the responses of all the sections, without distortion.

We have introduced a rational measure of the level of adaptation A, the inverse of the sensitivity, as usually defined. It is defined as being equal to the intensity of illumination with which the specimen would find itself in equilibrium. Our measurements of the variations of A in the various programs have permitted us to conjecture an equation describing the general functional relation between I and A, thus enabling us to chart the course of A for any program. Inspection of the growth outputs in the various programs then showed that there exists a significant correlation between the v outputs and the subjective intensity $i = I/A$, suggesting that the v output in the general case might be constructed by a superposition principle from a hypothetical output v_1 in response to a unit subjective stimulus. This notion has been subjected to several qualitative and one semi-quantitative test, which seem to support it.

Up to this point our analysis has been purely formal. We have refrained from giving the variables A and i a meaning in terms of concentrations of hypothetical substances and rates of hypothetical reactions. Our aim has been merely that of creating a conceptual framework

permitting us to describe the workings of the system quantitatively, and not to load this frame with prejudices as to the actual mechanism. However, to help the imagination in the design of further experiments it may be desirable to show that simple models can be constructed exhibiting at least some of the properties of our system.

In designing models we may conveniently start from the finding that the growth velocity at equilibrium is independent of the intensity of illumination, and that the net gain of growth after a transient change in intensity is zero. This suggests that the average velocity is determined by the rate of supply of a material M, manufactured in the mycelium and brought up with the protoplasmic stream. Let us assume that this material is supplied at a constant rate B, and that its concentration in the growing zone is $[M]$. The rate of conversion of this material into building blocks for the construction of the wall must now be governed by the intensity of illumination. The simplest way to accomplish this is to postulate the existence of an enzyme E whose activity is controlled by the light, and a rate of conversion of M into building blocks for the wall W, which is proportional to $[M]$ and to the enzyme activity. If the enzyme activity is held constant, $[M]$ reaches an equilibrium value such that the conversion rate of M into W equals the rate of supply B. This accounts for the constant growth rate, independent of the intensity of illumination. If the intensity changes transiently the enzyme activity changes transiently, and so does $[M]$, but it returns to its original value after the transient has passed. This accounts for the zero net gain in growth produced by a transient change in illumination. This simple model, while accounting for these qualitative features of the responses, fails when the quantitative aspects are concerned. Calculation shows that it does not give the logarithmic dependence of the response on the stimulus size, nor the exponential course of the dark adaptation. To account for these, we may postulate a more complex system illustrated in Fig. 11. This system involves two enzymes, E and E', the activity of both of which are light controlled. The activity of E adjusts slowly to changes in light intensity, with the time constant of 3.8 minutes of the real system. It converts the precursors M in a rapid reaction to inactive material X. The level of activity of this enzyme represents the level of adaptation, A, and it causes $[M]$ to be proportional to $1/A$. The activity of the second enzyme E' adjusts instantaneously to any changes in intensity. This enzyme converts the precursors M in a slow reaction into the wall material W at a rate proportional to its activity (therefore to I), and to the con-

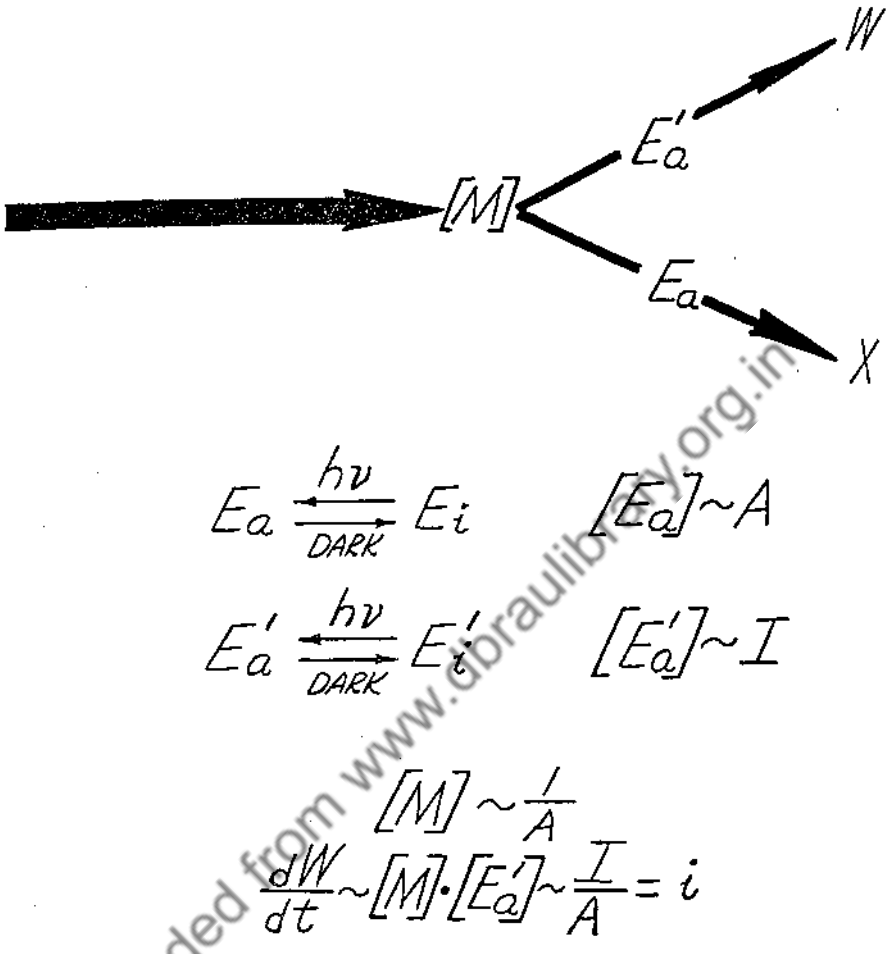


Fig. 11. A schematic diagram illustrating the model discussed in the text. The thickest arrow represents the constant supply of precursors M. The enzymes E and E' each exist in active forms (subscript a) and inactive forms (subscript i). The concentrations of these forms are controlled by light and dark reactions. The dark reaction for E has the time constant $b = 3.8$ min. $[E_a]$ is therefore proportional to the level of adaptation A. The dark reaction for E' is fast. $[E'_a]$ is therefore proportional to I. M is converted principally into the inert material X, the conversion into W (building blocks for the wall) constituting a minor side reaction. $[M]$ is determined by the constant supply rate and by $[E_a]$. It is proportional to $1/A$. The rate of production of W is proportional to $[M]$ and to $[E'_a]$, i.e. to $I \times 1/A = i$. The model does not account for the connection between $i(t)$ and $v(t)$.

centration of precursors, $[M]$ (therefore to $1/A$). The rate of production of building blocks W is therefore proportional to I/A, i.e. to what we have called the subjective intensity.

It is not claimed that this model is anything more than an illustration of the formal relations we have inferred from our experiments. Many

other models could be devised to serve the same purpose, and there would be little ground for preferring one to the others. The principal thing that suggests itself from a consideration of such models is a necessity of assuming in the chain of action starting at the light input a bifurcation, one branch leading to the setting of the level of adaptation and the other branch utilizing the light input directly so as to compare the instantaneous value of the intensity with the level of adaptation. Almost certainly this means that the system must be governed by more than one time constant, although only one has been revealed by the experiments here reported. These represent only a crude beginning. The most obvious extension will be the study of the limiting cases of light saturation and complete dark adaptation. After the normal pattern has thus been mapped out, one may hope to gain further insight from the study of abnormal situations by changing the biochemical environment or the genetic background.

We will conclude this presentation with a restatement of the problem in very general terms.

Basic to the functional organization in the living world is the ability of the individual cells to respond to stimuli. By this is meant that the stimulus controls the release of metabolic energy into a particular channel in an amount often disproportionately large compared to the stimulus. This characteristic of living cells used to be considered not only as one of the essential attributes of life but also as one of the features which distinguishes the living from the non-living world. With the advent of modern physics and its technology we find ourselves surrounded on every hand with physical stimulus-reaction systems which answer exactly the general definition of such systems, from the doorbell to the most complex electronic control systems. While life has thus been deprived of one of its prerogatives by the advance of technology it is still true that none of the stimulus-reaction chains of living organisms have been fully interpreted, and this despite very great efforts which have been expended in this direction. In our opinion the question whether or not the functional properties of any one cell can be fully interpreted in physical chemical terms is crucial for clarifying the relation between the physical sciences and biology. Notwithstanding the great successes of biochemistry we believe that the true relation between these two sciences is still obscure and will remain so until a suitable system has been found for which the analysis can be carried far beyond its present limitations.

X. SUMMARY

1. A general argument is presented indicating the theoretical and experimental advantages of graded versus threshold stimulus-reaction systems.

2. The general characteristics of normal growth and of the light growth responses of the sporangiophores of Phycomyces in stage IVb are described.

3. The interrelations between the phototropic and the light growth response are discussed. It is pointed out that current theories fail to account for the fact that the tropic response is limited to a region near the base of the growing zone.

4. Short sections of the growing zone have been stimulated and the growth responses measured. The results suggest that the responses, both with respect to growth and with respect to changes in the level of adaptation, are confined to the stimulated region.

5. It is shown that the direction of growth is in an *indifferent* phototropic equilibrium when the resultant of the illumination vectors (directed from specimen to light source) is zero. The equilibrium is *stable* when the resultant and the axis of the specimen are parallel. It is *unstable* when they are antiparallel.

6. The level of adaptation A is defined as the "equivalent intensity," i.e. the intensity with which the specimen will find itself in equilibrium.

7. A procedure is given for experimentally determining the level of adaptation.

8. The changes in A occurring as a result of various illumination programs are determined by this procedure.

9. A differential equation is given describing the general functional relationship between $I(t)$ and $A(t)$.

10. Arguments are presented suggesting that the growth output $v(t)$ stands in a linear functional relationship to the subjective intensity $i(t) = I(t)/A(t)$. This assumption is subjected to a quantitative test.

11. A chemical model is described illustrating the formal relations deduced from the observations.

BIBLIOGRAPHY

Blaauw, A. H. 1909. Die Perzeption des Lichtes. *Rec. trav. bot. Neerl.* 5, 209-372.

Blaauw, A. H. 1914. Licht und Wachstum I. *Zeit. f. Bot.* 6, 641-703.

Blaauw, A. H. 1918. Licht und Wachstum III. *Medd. Landbouwhoogeschool Wageningen 15*, 91-203.

Blaauw, A. H., and W. van Heyningen. 1925. The radium growth response of one cell. *Proc. Roy. Ac. Sci. Amsterdam 28*, 403-417.

Buder, J. 1918. Die Inversion des Phototropismus bei Phycomyces. *Bot. Ber. 36*, 104-105.

Buder, J. 1920. Neue phototropische Fundamentalversuche. *Bot. Ber. 38*, 10-14.

Buder, J. 1946. Uebersicht über Ergebnisse einiger noch ungedruckter Arbeiten aus den Botanischen Anstalten der Universität Breslau (mimeographed private communication).

Castle, E. S. 1929. Dark adaptation and the light growth response of Phycomyces. *J. Gen. Physiol. 12*, 391-400.

Castle, E. S. 1930. Phototropism and the light-sensitive system of Phycomyces. *J. Gen. Physiol. 13*, 421-435.

Castle, E. S. 1931. The phototropic sensitivity of Phycomyces as related to wavelength. *J. Gen. Physiol. 14*, 701-711.

Castle, E. S. 1933. The physical basis of the positive phototropism of Phycomyces. *J. Gen. Physiol. 17*, 49-62.

Castle, E. S., and A. J. M. Honeyman. 1933. The light growth response and the growth system of Phycomyces. *J. Gen. Physiol. 18*, 385-397.

Castle, E. S. 1942. Spiral growth and reversal of spiraling in Phycomyces and their bearing on primary wall structure. *Amer. J. Bot. 29*, 664-672.

Errera, L. 1884. Die grosse Wachstumsperiode bei den Fruchttraegern von Phycomyces. *Bot. Zeitung 52*, 496-503.

Frey-Wyssling, A., and K. Mühletaler. 1950. Der submikroskopische Feinbau von Chitinzellwänden. *Vierteljahresschrift naturf. Ges. Zürich 95*, 45-52.

Hagins, W. A., and W. A. H. Rushton. 1953. The measurement of rhodopsin in the decerebrate albino rabbit. *J. Physiol. 120*, 61P.

Hartline, H. K., and P. R. McDonald. 1947. Light and dark adaptation of single photoreceptor elements in the eye of Limulus. *J. Cell. Comp. Physiol. 36*, 225-254.

Hartline, H. K., H. G. Wagner, and E. F. MacNichol, Jr. 1952. The peripheral origin of nervous activity in the visual system. *Cold Spring Harbor Symp. Quant. Biol. 17*, 125-141.

Hecht, S. 1918-19. Sensory equilibrium and dark adaptation in *Mya arenaria*. *J. Gen. Physiol. 1*, 545-558.

Hodgkin, A. L., A. F. Huxley, and B. Katz. 1952. Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol. 116*, 424-448.

Mueller, C. G. 1954. A quantitative theory of visual excitation for the single photoreceptor. *Proc. Natl. Acad. Sci. 40*, 853-863.

Oort, A. J. P. 1931. The spiral growth of Phycomyces. *Proc. Roy. Acad. Sci. Amsterdam 34*, 564-575.

Oort, A. J. P. 1932. Die Wiederherstellung der Empfindlichkeit nach einem Lichtreiz. *Proc. Roy. Ac. Sci. Amsterdam 29*, 5-47.

Rushton, W. A. H., and R. D. Cohen. 1954. Visual purple level and the course of dark adaptation. *Nature 173,* 300-302.

Tollenaar, D. 1921. Dark growth responses. *Proc. Roy. Ac. Sci. Amsterdam 26,* 378-379.

Tollenaar, D., and A. H. Blaauw. 1921. Light and dark adaptation of a plant cell. *Proc. Roy. Ac. Sci. Amsterdam 24,* 1-16.

Wald, G. 1954. On the mechanism of visual threshold and visual adaptation. *Science 119,* 887-892.

Wassink, E. C., and M. A. Bouman. 1947. Can phototropism be initiated by a one-quantum-per-cell process? *Enzymologia 12,* 193-197.

Waterman, T. H., and C. A. G. Wiersma. 1954. The functional relation between retinal cells and optic nerve in Limulus. *J. Exp. Zool. 126,* 59-86.

Westphal, W. H. 1947. *Physik,* p. 464. Springer, Berlin and Heidelberg.

II. THE CYTOLOGY OF NUCLEAR RNA

BY HEWSON SWIFT, LIONEL REBHUN, ELLEN RASCH, AND JOHN WOODARD [1]

Some fifteen to twenty years have elapsed since the early work of Caspersson, Schultz, Brachet, and others first clearly suggested that nucleic acids were related to growth and biosynthesis in cells. Since that time many ways have been suggested in which nucleic acids may take part in cell processes: they may be energy donors or electron acceptors in peptide bond synthesis; they may serve a protective function for the genes during self-duplication; or they may possess a template structure on which the building blocks of proteins may be ordered and assembled.

Many of the earlier concepts, such as that of RNA-DNA interconversion in development or mitosis, have been found untenable. But the basic assumption, that nucleic acid plays a necessary role in protein synthesis, is now being put on a sound biochemical basis, for example, by the work of Gale and Folkes (1954), Webster (1955), Allfrey et al. (1953), and Zamecnik and Keller (1954). Evidence is now accumulating that ribonucleotides may effect the incorporation of amino acids into specific proteins, and that proteins in some cases may go through a step in their formation in which they are bound to RNA before they are released as tissue proteins in the cell.

These findings are largely biochemical, and much of the work has been done on *in vitro* systems. It also seems essential to place these chemical components in relation to the morphological framework of the cell. It is becoming increasingly clear, for example, in regard to mitochondria, that biochemical properties and structure are intimately related.

This paper discusses a few morphological aspects of RNA in several cell systems, particularly in respect to the nucleus. The amounts of RNA have been estimated in growing and synthesizing cells by dye binding and microphotometry. Certain changes in fine structure of

[1] Whitman Laboratory, University of Chicago. Supported by grants from the U.S. Public Health Service, and the Abbott Memorial Fund. Dr. Woodard is a postdoctoral fellow, U.S. Public Health Service. The authors gratefully acknowledge the kindness of Dr. Ruth G. Kleinfeld in providing data on rat liver regeneration, and to Dr. Lawrence Herman, Dr. James Lash, and Mr. Eubert Daniel for preparing some of the microscope slides and electron micrographs.

the nucleus have been tentatively correlated with changes in nucleic acid content. All tissues for photometry were fixed in acetic acid-alcohol and stained with azure B (Flax and Himes, 1952). Several variables tend to upset the stoichiometry of dye-nucleic acid binding (Swift, 1955). In the present studies, however, absorption curve analysis

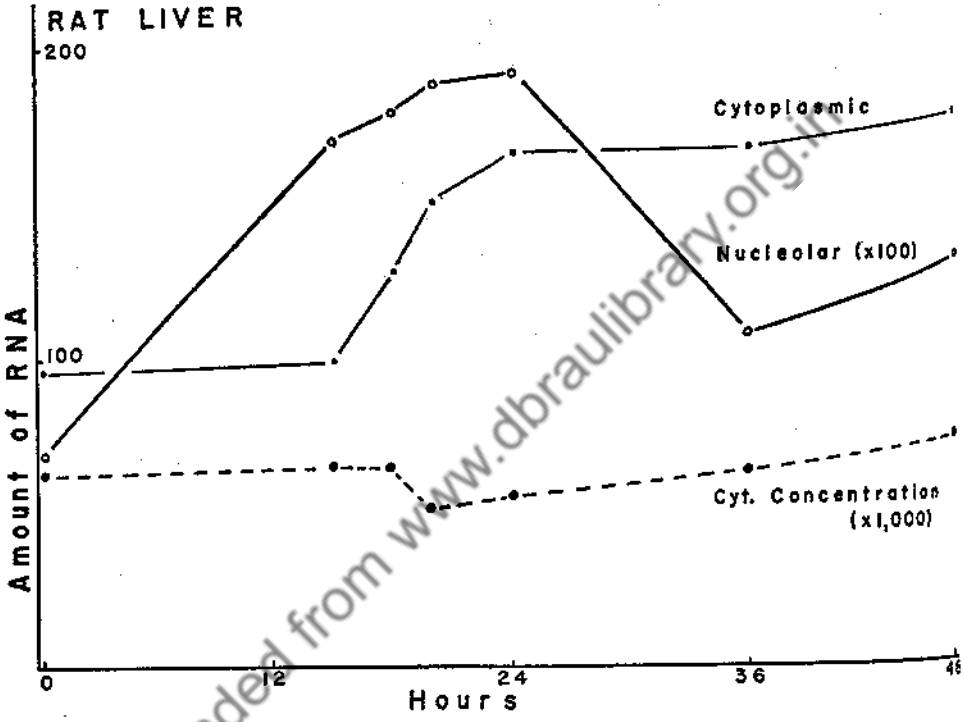


Fig. 1. Cytoplasmic and nucleolar RNA in rat liver regeneration after partial hepatectomy. Each point represents the mean of from 25 to 65 measurements, on sections stained with azure B (.025%). Cytoplasm was measured with the two wavelength method at 515 and 534 mμ, and values were corrected for distributional error to 550mμ. Nucleoli were measured at 550 mμ, so that cytoplasmic and nucleolar values are comparable. Cytoplasmic concentration is plotted as the corrected extinction at 550 mμ, per micron section thickness.

showed Beer's law to hold, and dye specificity was checked with ribonuclease. Areas of cells from 2 to 5 microns in diameter were measured at various wavelengths. Where dye distribution was markedly irregular the two wavelength method was used. The photometric techniques employed are discussed by Swift and Rasch (1956). Standard techniques were used for electron microscopy, including fixation in buffered osmic acid (Palade, 1952), and methacrylate imbedding.

I. NUCLEOLI

Nuclear RNA occurs in association with three morphologically distinct fractions: the nucleolus, the chromosomes, and the nuclear membrane. The nucleolus is the most obvious RNA-containing component of the nucleus. It is often a sensitive index of cell activity, showing marked changes in the early stages of cell growth preceding cytoplasmic RNA synthesis, as for example in liver regeneration (Fig. 1). It is thus natural that the nucleolus should be implicated in nuclear RNA

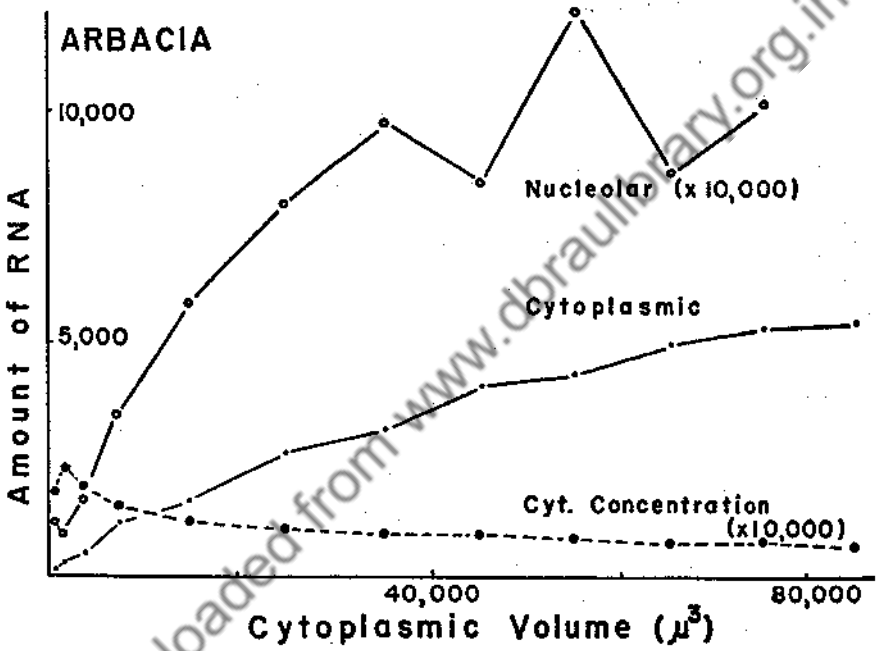


Fig. 2. Cytoplasmic and nucleolar RNA in oocytes of *Arbacia punctulata*. Each point represents the mean of from 4 to 7 measurements on sections stained with azure B (.025%) made at a wavelength of 550 mμ. Oocytes in the largest size class were undergoing the first maturation division, and nucleoli were absent. Concentration is plotted as extinction per micron section thickness.

synthesis, and further that cytoplasmic RNA should be considered as having its origin in the nucleus (Caspersson, 1941; Jeener and Szafarz, 1950). Several recent findings have cast doubt on this theory. Base ratios of RNA's isolated from nuclei are different from those of the cytoplasm (Davidson, 1953; Moldave and Heidelberger, 1954), and incorporation studies strongly suggest that nuclear and cytoplasmic fractions are independent (Barnum et al., 1953). Also, as shown by

Brachet and Szafarz (1953), incorporation of labeled orotic acid into cytoplasmic RNA continues in *Acetabularia* after removal of the nucleus.

Information on nuclear-cytoplasmic relations can also be obtained by examining total amounts of RNA in nucleolus and cytoplasm of individual cells undergoing protein synthesis. Measurements are shown for regenerating rat liver (Fig. 1), for growing oocytes of sea urchin (Fig. 2) and clam (Fig. 3), and for forming *Tradescantia* pollen

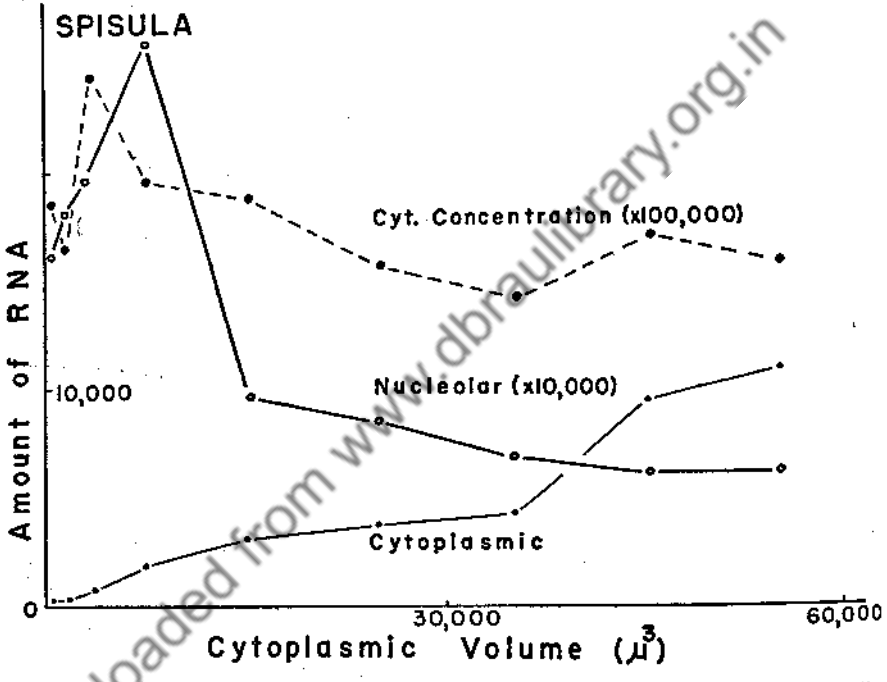


Fig. 3. Cytoplasmic and nucleolar RNA in oocytes of the surf clam *Spisula solidissima*. Each point represents the mean of from 4 to 13 measurements. Other details as in Fig. 2.

grains (Fig. 4). It is impossible from these data to describe any simple relation between the nucleolus and the various RNA-containing fractions of the cytoplasm. In three cases, namely, those of liver, clam oocyte, and pollen grain, the nucleolar RNA increased markedly during the early growth stages, and then dropped abruptly, even though the total cytoplasmic RNA continued to increase. This is particularly obvious in the *Tradescantia* pollen where the nucleolus of the vegetative nucleus disappeared completely at least a full day before the pollen was shed, during which time the total cytoplasmic RNA increased 30%. The

generative nucleus at this time also lacked a visible nucleolus. In *Arbacia* oocytes, however, the nucleolar RNA paralleled the cytoplasmic RNA, and continued to increase throughout the growth period and up to the first maturation division. The difference in nucleolar behavior between

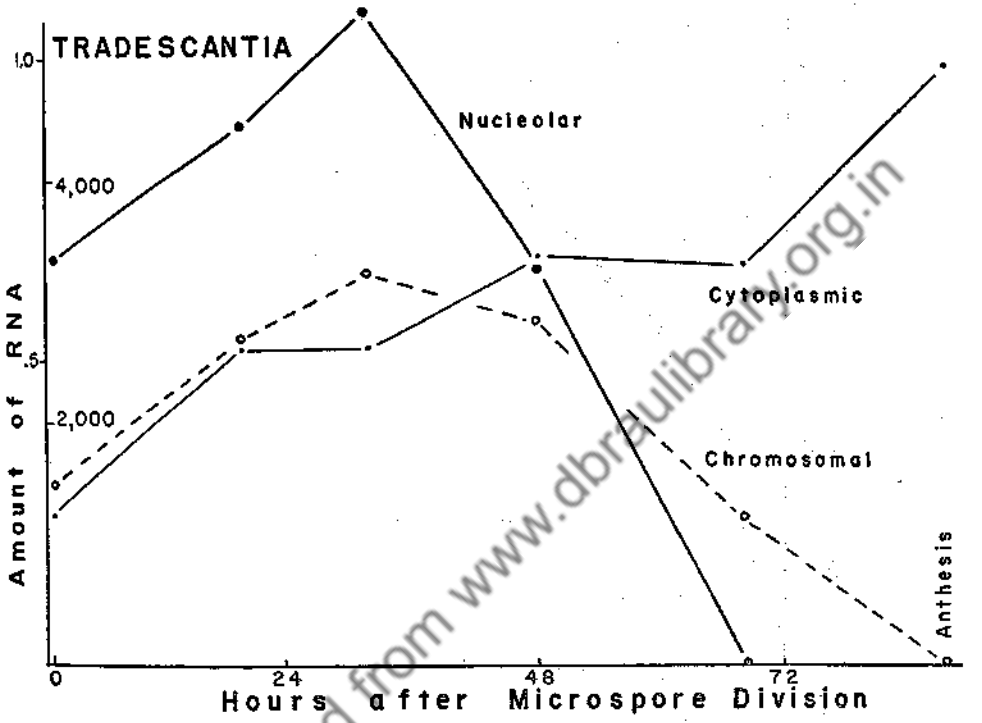


Fig. 4. Cytoplasmic, nucleolar, and chromosomal RNA in *Tradescantia paludosa* pollen. On the ordinate, right hand scale applies to cytoplasm, and left hand scale to nucleolus and chromosome measurements. Values cover the period from early interphase immediately after the microspore division, to the time of pollen shedding. Nucleolar and chromosomal measurements are for vegetative nucleoli only; there was no measureable RNA in chromosomes of the generative nuclei, and within 20 hours after the microspore division nucleoli in the generative nuclei were too small to measure. Values are means of from 10 to 15 measurements on sections stained with azure B (.025%). Cytoplasmic RNA was measured with the two wavelength method at 440 and 478 mμ, and values were corrected for distributional error. Nucleoli were measured at 500 and chromosomes at 600 mμ. All values have been corrected for a wavelength of 500 mμ, and are directly comparable.

Arbacia and the clam *Spisula* is evident in Plate I. Our data indicate that cytoplasmic RNA can be synthesized in some cases independently of the nucleolus. They do not support the concept that the nucleolus is directly involved in cytoplasmic RNA synthesis.

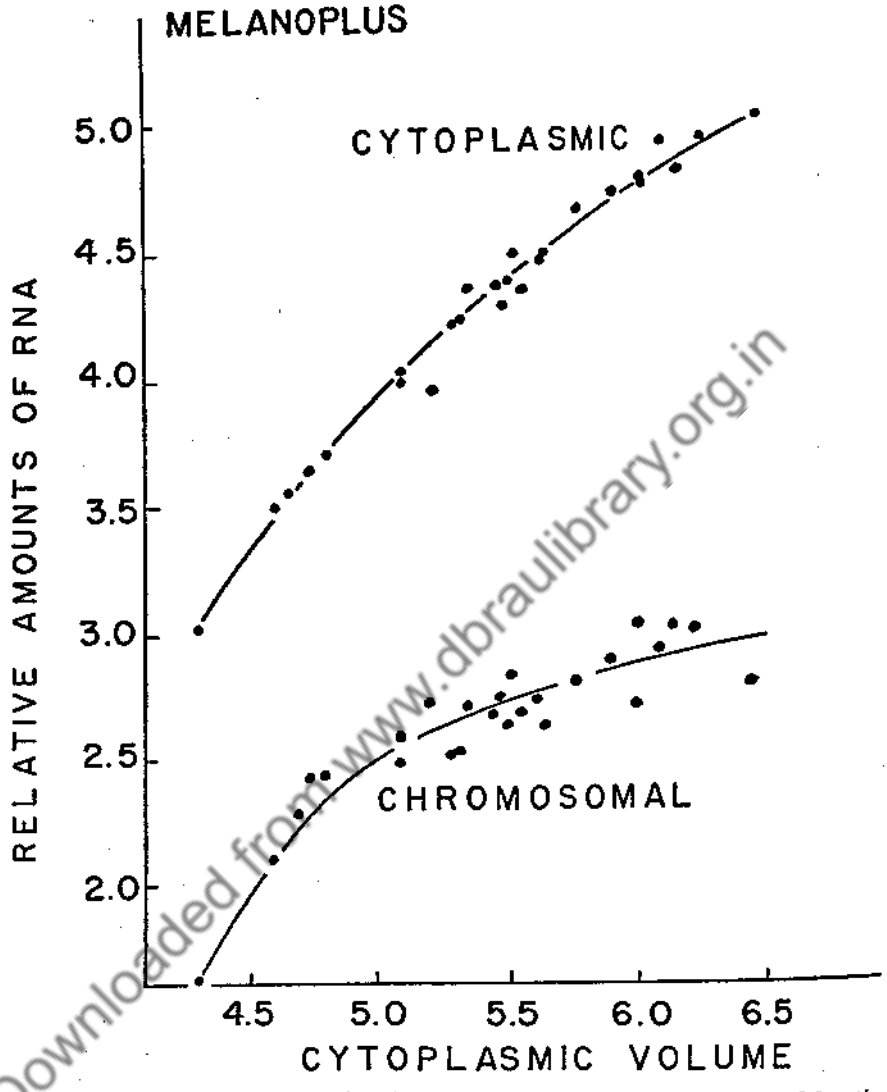


Fig. 5. Chromosomal and cytoplasmic RNA in oocytes of the grasshopper *Melanoplus differentialis*. The logarithm of total RNA is plotted against the logarithm of oocyte volume in μ^3. Points represent individual measurements made at 500 mμ on sections stained with azure B (.025%).

II. THE CHROMOSOMES

Several workers have described RNA as a component of mitotic or interphase chromosomes. Mirsky and Ris (1947) found a small amount of RNA associated with the residual protein from isolated chromosome threads of beef tissues. Kaufmann et al. (1948, 1951),

Flax and Himes (1951), and Jacobson and Webb (1952) have described RNA in mitotic chromosomes on the basis of various staining reactions used in conjunction with ribonuclease and desoxyribonuclease.

The chromosomes of many tissues, however, after DNA removal,

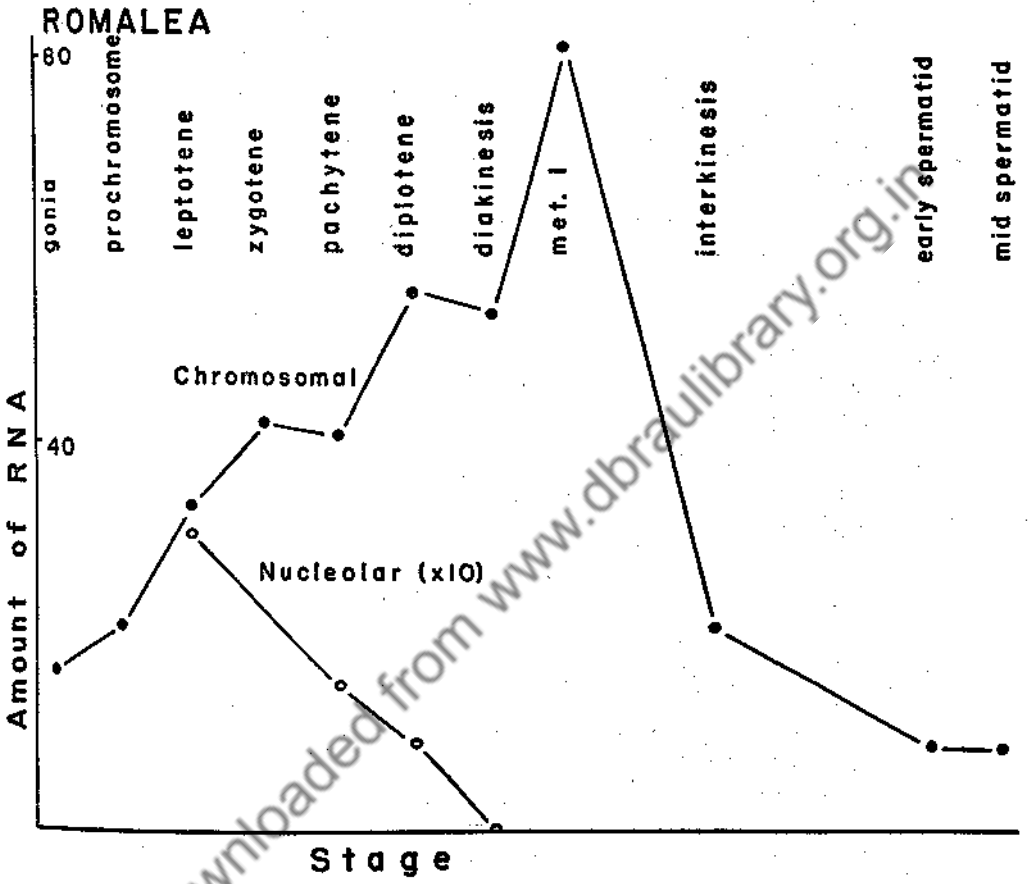


Fig. 6. Chromosomal and nucleolar RNA during spermatogenesis in the grasshopper *Romalea microptera*. Values represent the means of from 6 to 15 measurements made on sections stained with azure B (.25%). Chromosomal RNA was measured with the two wavelength method at 510 and 550 mμ, and was corrected for distributional error to 550 mμ. Nucleoli were measured at 550 mμ. Metaphase values necessarily included some spindle RNA, and are therefore too high.

do not stain appreciably with basic dyes, and thus no RNA is demonstrable. Also, where chromosomes undergo marked changes in protein-nucleic acid ratios, as in mitosis, the amounts of dye bound do not necessarily reflect nucleic acid content (Swift, 1953a, 1955). These factors have made studies on the RNA staining of mitotic chromosomes

difficult to evaluate. It is possible that RNA is a normal component of chromosomes, but that it may exist in a form not available for dye binding. This is supported by ultraviolet absorption studies (Rasch, cf. Swift, 1953b), which indicated the presence of considerable amounts of RNA in mitotic lily chromosomes, and also by Gay (1949) who found that RNA in interphase onion chromosomes became stainable after trypsin digestion.

In some tissues chromosomal RNA is readily available to basic dyes without any special treatment. The staining is, however, often faint in comparison with cytoplasmic or DNA staining and thus may be overlooked. In some tissues, however, such as *Drosophila* nurse cells or grasshopper oocytes, chromosomal RNA becomes intensely staining, so that it overshadows the DNA. Measurements on chromosomal RNA are shown for grasshopper oocytes (Fig. 5 and Plate II) and spermatocytes (Fig. 6 and Plate III), for *Tradescantia* pollen grains (Fig. 4), and for *Ambystoma* liver during re-feeding after starvation (Table I and Plate IV).

TABLE I. Relative amounts of RNA in larval *Ambystoma* liver cells during re-feeding after 3 weeks of starvation.*

Time in hrs.	Chromosomal RNA mean	S.E.	*n*	Nucleolar RNA mean	S.E.	*n*	Cytopl. conc. E/μ	Cytopl. RNA per cell
0	8.59	± .50	15	1.47	± .07	11	.034	70
12	10.46	± 1.14	10	1.88	± .10	10	.042	182
36	19.74	± 1.18	20	4.56	± .23	10	.048	374

* Slides were stained with azure B (.25%), and were measured at a wavelength of 550 mμ. Values are expressed as EV/d, where E is the extinction, d section or structure thickness, and V the volume of nucleolus, nucleus, or cell. Cell volume was determined by the Chalkley method. Data are from six larvae, all about 3 cm in length.
S.E. = Standard error; n = number of cases.

From these data several conclusions can be drawn. (1) The RNA of the chromosomes, like the nucleolus, appeared to show marked changes in amount during cell growth. (2) In liver and pollen grains the chromosomal and nucleolar RNA showed approximately parallel changes. This also appeared to be true of the *Ambystoma* thyroid cells during stimulation by thyrotropic hormone, shown in Plate IV, although the nucleoli in these cells were too small for measurement. No

correlation between chromosomal and nucleolar RNA was found in grasshopper spermatogenesis, where chromosomes showed an abrupt increase in stain as the nucleolus disappeared. (3) Chromosomal RNA paralleled the cytoplasmic RNA during grasshopper oocyte growth, but showed no correlation in the pollen grain. (4) Meiotic chromosomes showed a marked loss in RNA following metaphase, as also described by Jacobson and Webb (1952). A similar RNA loss is apparent in some moth oocytes (Ris and Kleinfeld, 1952). These findings are preliminary and in need of confirmation by ultraviolet absorption. They do indicate, however, that chromosomal RNA is an important component of the nucleus. It was of greater magnitude than nucleolar RNA in the four tissues measured. Like the nucleolus, it appears to bear no direct relation to the total cytoplasmic RNA.

In nuclei of *Ambystoma* thyroid and grasshopper oocytes, much of the DNA-containing chromosomal material is condensed into clearly visible blocks. In the oocytes these are the expanding diakinetic bivalents. In *Ambystoma* they are interphase "chromocenters" and appear commonly in many tissues. When chromosomal RNA begins to form in such nuclei it appears clearly as an irregular rim surrounding the condensed chromosomal material. This relationship is also evident in electron micrographs of *Ambystoma* thyroid nuclei. Before stimulation the clumped chromatin appears as uniformly electron dense, with a very fine filamentous texture. At the time chromosomal RNA forms, these areas are surrounded by large granular regions containing particles of widely varying size which frequently show a linear arrangement (Plate V). Similar structures were evident in *Ambystoma* liver nuclei. Because of the marked similarity between the cells under the electron microscope and after RNA staining (Plate IV) it seems likely that chromosomal RNA is associated with these large granular regions. In the chromosomes of amphibian oocytes, RNA has been associated with somewhat similar granules in the lampbrush loops (Gall, 1955).

III. NUCLEAR MEMBRANE

In cells preparing for synthetic activity, cytoplasmic RNA often first appears as a ring surrounding the nucleus, later becoming more widely distributed through the cytoplasm. This distribution occurs, for example, in the pallial layer of young oocytes of many species. It is also evident in the *Ambystoma* thyroid and liver nuclei shown in Plate IV. The perinuclear ring of RNA has been widely considered (e.g. by Caspersson, 1936, 1941) as evidence for nuclear-cytoplasmic interac-

tion, and possibly as representing nucleoprotein exchange from the nucleus to cytoplasm. Since evidence is accumulating against this view, it is of interest to examine this RNA fraction particularly in regard to its site of origin.

Cytoplasmic RNA is certainly a heterogenous fraction. RNA has been associated with mitochondria (Schneider, 1946) and the Golgi apparatus (Schneider and Kuff, 1954). Probably the largest fraction, and the one primarily responsible for basophilia has been called the microsomal fraction or the endoplasmic reticulum. Neither of these names is generally applicable for the wide variety of basophilic structures occurring in tissues. Thus the more general term, ergastoplasm, is used here.

In oocytes of the clam *Spisula,* during most of the growth period, intensely basophilic plate- or brick-like structures lie close to or against the nuclear membrane. Often further out in the cytoplasm are basophilic cup-shaped or spheroidal structures, frequently bordering or enclosing clear areas of cytoplasm free from basophilia. From a study of these structures under a light microscope it seems probable that they arise in conjunction with the nuclear membrane, move peripherally where they enlarge by becoming cups or spheres, and eventually disintegrate (Plate I).

The formation of cytoplasmic RNA is markedly different in the thyroid cells of *Ambystoma.* In adult animals the glandular epithelium is low cuboidal, containing almost no cytoplasmic RNA. Within two to seven days following treatment with thyroid-stimulating hormone, the cell height increases markedly, and RNA appears first as a cap and later usually as a cone, on the distal margin of the nucleus (Plate IV). In later stages the cone is disrupted as secretion products appear.

The nuclear membrane in a number of tissues, as viewed under the electron microscope, characteristically contains numerous electron dense annuli, with dimensions characteristic of the tissue or species (Callan and Tomlin, 1950; Gall, 1954; Afzelius, 1955; Rebhun, 1956). In many preparations annuli appear to be made up of small densely packed particles, in some cases also surrounded by a few scattered particles of similar dimensions. Nuclear membranes from oocytes of the land snail *Otala* are shown in Plate VI. When the annuli in these preparations are transected in sectioning they appear as two small parallel lines, traversing both osmophilic layers of the nuclear membrane, and projecting slightly into the cytoplasm. On the nuclear side they can be seen to pro-

ject often a considerable distance into the nucleus, as poorly defined regions containing the usually particulate and filamentous texture of the nucleus in slightly higher concentration than the surrounding regions. The annuli thus apparently occur where rods or cylinders of nuclear material terminate at the nuclear membrane (Plate VI, 2, 3, 4). Somewhat similar findings for echinoderm oocytes have recently been published by Afzelius (1955). In sections such as shown in Plate VI, 2, the inner and outer layers of the nuclear membrane often appear to traverse the annuli without interruption. In other cases they are pinched together in the annular region, and occasionally appear to be interrupted, apparently forming an opening through the annular center.

In both *Otala* and *Spisula* oocytes, structures identical to the nuclear membrane lie parallel to it in the cytoplasm. These vary in size from pieces containing only a few annuli (Plate VI, 2) through intermediate-sized stacks of lamellae (Plate VII, 1 and Plate VIII, 1) to comparatively large areas up to 3 microns thick and 10 microns long. The largest structures, found only in *Spisula,* are without doubt the basophilic bricks or plates of ergastoplasm seen in *Spisula* under the light microscope. These lamellae appear banded, as does the nuclear membrane when the section transects an annulus. In the lamellae, however, the annuli appear to be more closely and evenly packed. In some cases the annuli appear to traverse several lamellae, and may be responsible for holding adjacent lamellae together in a vertical orientation (Plate VII, 1). In tangent view it is often difficult to distinguish lamellae from the nuclear membrane. Plate IX, 1 shows a stack of lamellae apparently twisted so that they are cut at progressively smaller angles; annuli are apparent in the last lamella at the bottom. Plate IX, 2 shows what is probably a tangential section through a single lamella, demonstrating a hexagonal packing of annuli. In Plate IX, 3 a section tangent to the nuclear membrane is shown. In one region a small lamella appears to overlap the membrane.

Lamellae are frequently found to be swollen at the ends into small vesicles lined with particles. These vesicles are variable in size, but usually smaller in *Otala* than in *Spisula.* It is possible that they are artifacts produced by osmotic changes in the cell on fixation. Since a number of the vesicles are filled with electron-dense material, however, this seems unlikely. As shown in Plate VII, 1, vesicles form between the membranes of a single lamella. In the spherical or cup-shaped ergastoplasmic region, evidence of annuli usually is absent (Plate VII, 2).

Granules are distributed more or less evenly along the lamellae, and they resemble the typical ergastoplasm of, for example, mammalian pancreas (Weiss, 1953).

In *Ambystoma* thyroid the region of nuclear membrane and forming ergastoplasm appears as a complex and irregular array of membranes. In a few cases lamellae have been pulled away from the nucleus during preparation of the tissues, probably due to methacrylate shrinkage during plasticizing. In such cases there appears to be an intimate relation in a few areas between nuclear membranes and ergastoplasm, such that the first sac of the ergastoplasm is continuous with the nuclear membrane (Plate X, 1 and 2). Our photographs are as yet insufficient to make this relationship clear, but in general they support the concept of Watson (1955) that ergastoplasm of this type may form from outpocketings of nuclear membrane.

Nuclear-cytoplasmic exchange is also suggested in the two cells shown in Plate X, 3 and 4. Blebs in the outer layer of the nuclear membrane are visible in certain cells of the ovotestis of *Otala,* and vesicles of similar appearance are evident in the cytoplasm. Somewhat similar structures have been reported by Gay (1955). Nerve cells from the cerebral ganglion of *Otala* (Plate X, 4) were found to contain numerous large vesicles, which also appear to arise from the outer layer of the membrane. Although nuclear membrane annuli extend through to the outer layer, they are not apparent on the walls of the formed vesicles. It seems likely that during vesicle formation the particles in the annuli spread to form the arrangement of granules shown. This arrangement is consistent within one vesicle, but variable between them, suggesting that the granules either undergo a series of changes, or else show some type of specificity.

IV. CONCLUSIONS

Cytochemical studies on the RNA of nucleoli and chromosomes failed to demonstrate any simple relation with cytoplasmic RNA. In two cases the nuclear RNA fractions were found to decrease or disappear even during periods of rapid cytoplasmic RNA accumulation. This strongly suggests that in these tissues RNA is synthesized in the cytoplasm. It is possible that the RNA of the nucleolus and chromosomes is active in synthesis within the structures in which they occur, and is not transferred to any other cell region (cf. Worley, 1946). The basophilic structures in *Spisula* contain high concentrations of RNA organized into a complex three-dimensional lattice. Increase in size of these struc-

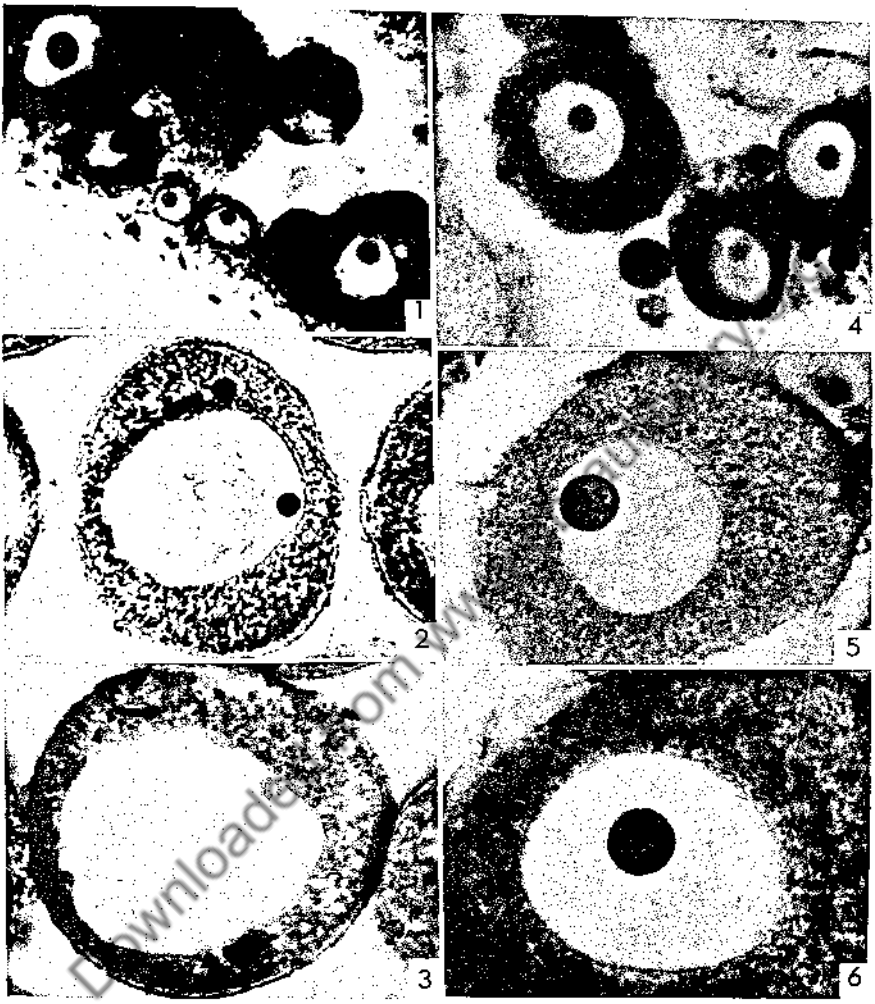


PLATE I. Stages in oocyte growth (×1000). 1-3. *Spisula solidissima*. 4-6. *Arbacia punctata*. All sections have been stained with azure B after treatment with desoxyribonuclease. Note the marked reduction in size of the basophilic nucleolus in later oocytes of *Spisula*, in contrast to the large nucleoli of late *Arbacia* oocytes. Intensely basophilic brick-like or spheroidal bodies are visible in the cytoplasm of *Spisula* oocytes.

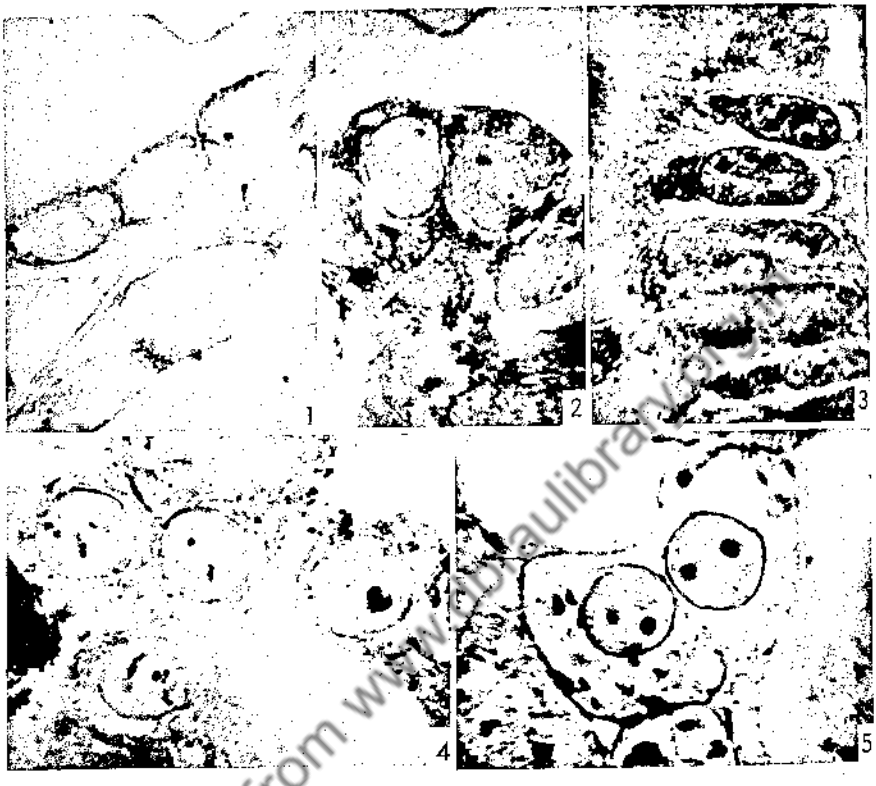


PLATE IV. Tissues of *Ambystoma tigrinum* stained with azure B after treatment with desoxyribonuclease (Fig. 3 untreated) (×1000). 1-3. Adult thyroid gland. 4-5. Liver cells from 3 centimeter larvae. 1. Epithelium of thyroid before stimulation. The cells are low cuboidal with barely visible nucleoli, and only a small amount of faintly staining cytoplasm. 2. Epithelium of thyroid 7 days after daily injection of thyrotrophic hormone (Parke, Davis, 0.1 mg). Cells have increased in height with increase in total cell, as well as cytoplasmic, volume. Nucleoli are enlarged, and the nucleus is filled with chromosomal RNA appearing as irregular rims surrounding condensed DNA-containing material. 3. Epithelium of thyroid 15 days after stimulation. In this preparation DNA has not been removed and it is characteristically clumped into basophilic masses. Caps of RNA-containing material are visible at distal margins of nuclei (cf. Plate V,3). 4. Liver parenchyma of larva after 3 weeks' starvation. Nucleoli are usually small, faintly staining, and in many cases, markedly irregular in shape. An unusually large nucleus is visible at right. Nuclei show little or no staining, and the cytoplasm is only faintly basophilic. 5. Larval liver parenchyma 36 hours after re-feeding. Nucleoli are very large and strongly basophilic. Nuclei are filled with RNA-staining material, and the nuclear membrane is strongly stained. Basophilic inclusions are abundant in the cytoplasm.

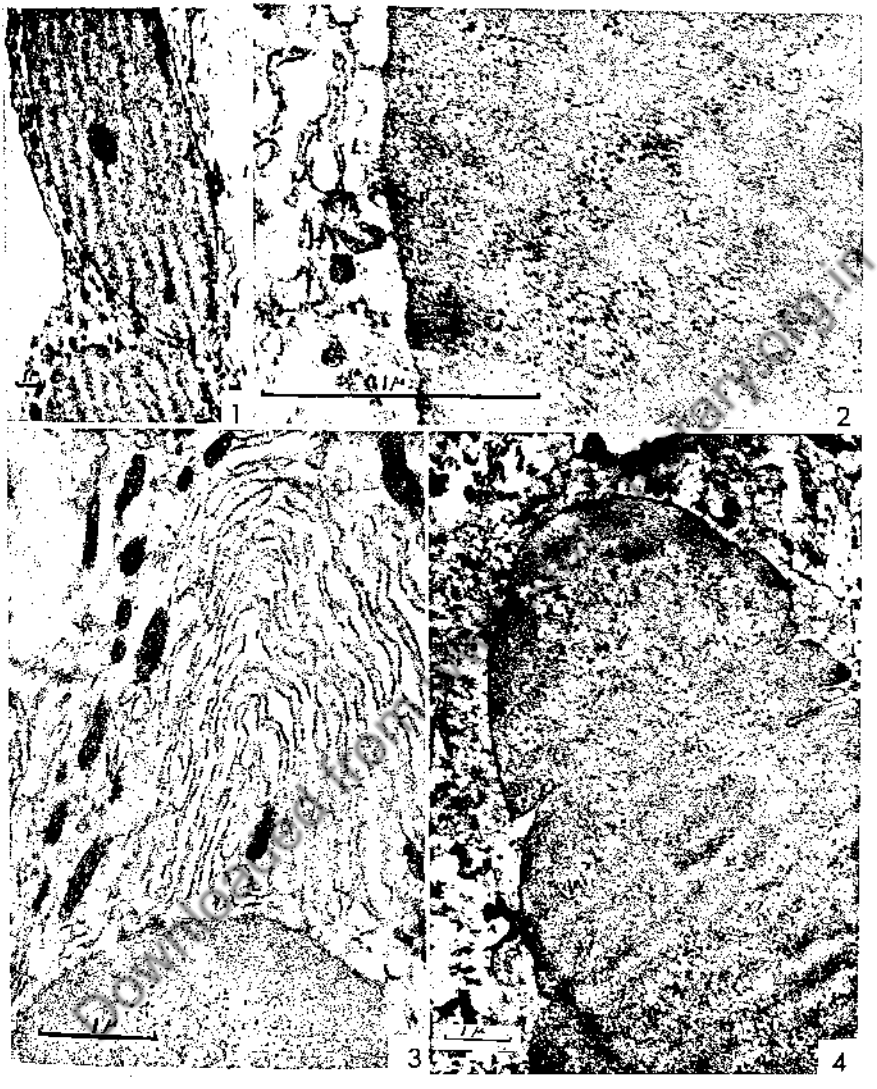


PLATE V. Electron micrographs of *Ambystoma tigrinum* thyroid. 1. Unstimulated cell (cf. Plate IV,1). 2. Seven days after start of daily injections of thyrotrophic hormone (Parke, Davis, 0.1 mg). A granular component is visible in the nucleus. 3. Fifteen days after start of daily injections, showing cap of ergastoplasm adjacent to nucleus (cf. Plate IV,3). 4. Treatment as in Fig. 2. Note the granular component surrounding areas of less electron-dense chromosomal material. The convoluted nuclear membrane is typical of this stage.

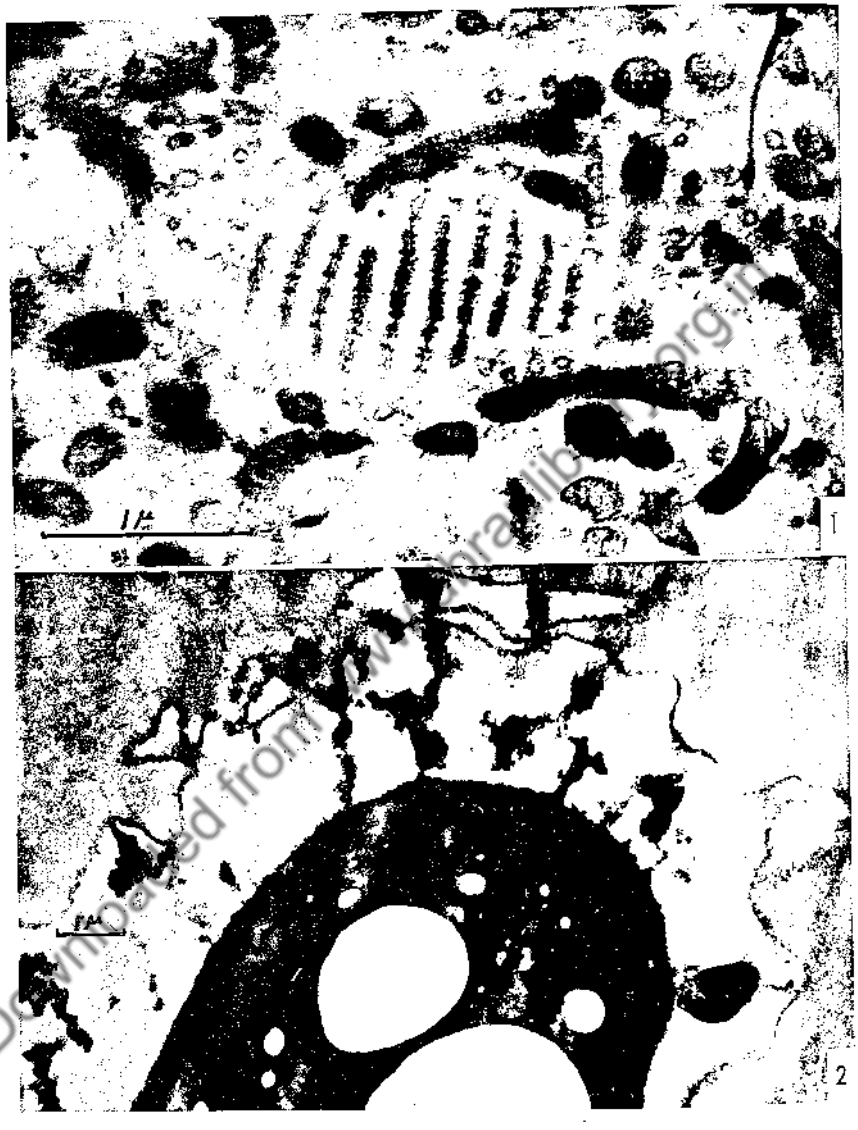


PLATE VIII. Electron micrographs of oocytes from the snail *Otala lactea*. 1. Small lamel region in cytoplasm. Lamellae contain annuli, and are vesiculate at the ends. 2. Young oocy showing large vacuolate nucleolus, with radiating chromosomes. Chromosomes are intimat associated with the convoluted nuclear membrane.

PLATE IX. Electron micrographs of lamellae from *Spisula* oocytes, as seen in tangent sections. 1. A group of lamellae cut in cross section (top) and tangentially (bottom). Note annuli in the region where the cut is tangential. 2. Section through what appears to be a single lamella, showing hexagonal arrangement of annuli. Nuclear membrane is shown in cross section at bottom. 3. A section of nuclear membrane, in tangent section, apparently with an adhering small lamella in center.

PLATE X. Electron micrographs suggesting a relation between nuclear membrane and some cytoplasmic structures. 1, and 2. *Ambystoma tigrinum* thyroid, after 7 days of thyrotrophic hormone stimulation. The ergastoplasm has been displaced in preparation, revealing areas in which cytoplasmic lamellae apparently form a part of the nuclear membrane. 3. Portion of a cell from the ootestis of *Otala*, probably a young oocyte, showing blebs in outer layer of the nuclear membrane (arrow). Note vesicles of similar appearance in the cytoplasm. 4. Portion of giant neuron from *Otala* ganglion, showing annuli in nuclear membrane, and outpocketing (arrow) at outer layer. Vesicles of similar structure are numerous in the cytoplasm. (Tissue was treated with distilled water before fixation.)

tures may involve synthesis of RNA, but radioautographic studies are needed to demonstrate this. If so, then the way in which these structures form is of importance. Are new materials supplied from the nuclear side, and the lamellae moved out, or are cytoplasmic components organized into new outer layers? When lamellae are against the nucleus, the nuclear membrane is almost always intact below them, although in a few cases shingle-like overlaps have been found. Also, the smallest (presumably forming) lamellae, as in Plate VI, 2, are on the outer surface of a lamellar region. Both these observations suggest that lamellar growth involves an increase in RNA from the cytoplasmic and not the nuclear margin.

Also of interest is the disappearance of annuli when vesicles are formed from either the nuclear membrane or the lamellae, and the simultaneous appearance of numerous particles lining the single layered vesicle membranes. If these particles obtain their origin from the annuli, then a connection is suggested between the chromosomal material and the apparent sites of protein synthesis. It might also be suggested that lamellar formation of the type found in *Spisula* and *Otala* may afford a type of duplication of annuli, particularly in times of rapid cell growth, independent of the nucleus.

In our present state of ignorance, speculations are far too easy. A great deal of careful work needs to be done before the highly complex interaction of nucleus and cytoplasm is understood.

BIBLIOGRAPHY

Afzelius, B. A. 1955. The ultrastructure of the nuclear membrane of the sea urchin oocyte as studied with the electron microscope. *Exp. Cell Res. 8,* 147-158.

Allfrey, V., M. M. Daly, and A. E. Mirsky. 1953. Synthesis of protein in the pancreas. II. The role of ribonucleoprotein in protein synthesis. *J. Gen. Physiol. 157,* 157-175.

Barnum, C. P., R. A. Huseby, and H. Vermund. 1953. A time study of the incorporation of radiophosphorus into the nucleic acids and other compounds of a transplanted mouse mammary carcinoma. *Cancer Res. 13,* 880-889.

Brachet, J., and D. Szafarz. 1953. L'incorporation d'acide orotique radioactif dans des fragments nucléés et anucléés *d'Acetabularia mediterranea. Biochem. Biophy. Acta 12,* 588-589.

Callan, H. G., and S. G. Tomlin. 1950. Experimental studies on amphibian oocyte nuclei. I. Investigation of the structure of the nuclear membrane by means of the electron microscope. *Proc. Roy. Soc.* (London). *B, 137,* 367-378.

Caspersson, T. 1936. Uber den chemischen Aufbau der Strukturen des Zellkernes. *Scand. Arch. Physiol. 73,* Suppl. 8, 1.

Caspersson, T. 1941. Studien uber den Eiweissumsatz der Zelle. *Naturwiss. 29,* 33-43.

Davidson, J. N. 1953. Les nucléoproteines et la croissance des tissus. *Bull. Soc. Chim. Biol. 35,* 49-66.

Flax, M. H., and M. H. Himes. 1951. Changes in chromosomal ribonucleoprotein during mitosis. (Abstr.) *Anat. Rec. 111,* 465.

Flax, M. H., and M. H. Himes. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool. 25,* 297-311.

Gale, E. F., and J. P. Folkes. 1954. Effect of nucleic acids on protein synthesis and amino-acid incorporation in disrupted Staphylococcal cells. *Nature 173,* 1223-1227.

Gall, J. G. 1954. Lampbrush chromosomes from oocyte nuclei of the newt. *J. Morph. 94,* 283-351.

Gall, J. G. 1955. Problems of structure and function in the amphibian oocyte nucleus. *Soc. Exp. Biol. Symposia.* In press.

Gay, H. 1949. Cytochemical studies using proteolytic enzymes. (Abstr.) *Amer. J. Bot. 36,* 796-797.

Gay, H. 1955. Nucleo-cytoplasmic relations in salivary gland cells of *Drosophila. Proc. Nat. Acad. Sci. 41,* 370-375.

Jacobson, W., and M. Webb. 1952. The two types of nucleoproteins during mitosis. *Exp. Cell Res. 3,* 163-183.

Jeener, R., and D. Szafarz. 1950. Relations between the rate of renewal and the intracellular localization of ribonucleic acid. *Arch. Biochem. 26,* 54-67.

Kaufmann, B. P., M. R. McDonald, and H. Gay. 1948. Enzymatic degradation of ribonucleoproteins of chromosomes, nucleoli, and cytoplasm. *Nature 162,* 814-815.

Kaufmann, B. P., M. R. McDonald, and H. Gay. 1951. The distribution and interrelation of nucleic acids in fixed cells as shown by enzymatic hydrolysis. *J. Cell. Comp. Physiol. 38,* Suppl. 1, 71-94.

Marshak, A., and C. Marshak. 1953. Desoxyribonucleic acid in *Arbacia* eggs. *Exp. Cell Res. 5,* 288-300.

Mirsky, A. E., and H. Ris. 1947. The chemical composition of isolated chromosomes. *J. Gen. Physiol. 31,* 7-18.

Moldave, K., and C. Heidelberger. 1954. Intramolecular heterogeneity in nucleic acid biosynthesis. *J. Am. Chem. Soc. 76,* 679-684.

Palade, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med. 95,* 285-298.

Rebhun, L. I. 1956. Electron microscopy of basophilic structures of some invertebrate oocytes. I. Periodic lamellae and the nuclear envelope. *J. Biophysic. and Biochem. Cytol. 2,* 93-104.

Ris, H., and R. G. Kleinfeld. 1952. Cytochemical studies on the chromatin elimination in *Solenobia* (Lepidoptera). *Chromosoma 5,* 363-371.

Schneider, W. C. 1946. Intracellular distribution of enzymes. I. The distribution of succinic dehydrogenase, cytochrome oxidase, adenosine-tri-

phosphatase, and phosphorus compounds in normal rat tissues. *J. Biol. Chem. 165,* 585-593.

Schneider, W. C., and E. L. Kuff. 1954. On the isolation and some biochemical properties of the Golgi substance. *Amer. J. Anat. 94,* 209-224.

Swift, H. 1953a. Quantitative aspects of nuclear nucleoproteins. *Int. Rev. Cytol. 2,* 1-76.

Swift, H. 1953b. Nucleoproteins in the mitotic cycle. *Texas Rpts. Biol. Med. 11,* 755-774.

Swift, H. 1955. Cytochemical techniques for nucleic acids. In *The Nucleic Acids,* vol. 2, ed. by Chargaff, E., and J. N. Davidson. Academic Press, New York.

Swift, H., and E. Rasch. 1956. Microphotometry with visible light. In *Physical Techniques in Biological Research,* vol. 2, edited by A. W. Pollister and G. Oster. Academic Press, New York. In press.

Thomson, R. Y., F. C. Heagy, W. C. Hutchinson, and J. N. Davidson. 1953. The desoxyribonucleic acid content of the rat cell nucleus and its use in expressing the results of tissue analysis, with particular reference to the composition of liver tissue. *Biochem. J. 53,* 460-474.

Watson, M. L. 1955. The nuclear envelope, its structure and relation to cytoplasmic membranes. *J. Biophysic. and Biochem. Cytol. 1,* 257-270.

Webster, G. C. 1955. Nitrogen metabolism. *Ann. Rev. Plant Physiol. 6,* 43-70.

Weiss, J. M. 1953. The ergastoplasm. Its fine structure and relation to protein synthesis as studied with the electron microscope in the pancreas of the Swiss albino mouse. *J. Exp. Med. 98,* 607-618.

Worley, L. G. 1946. The Golgi apparatus—an interpretation of its structure and significance. *Ann. N.Y. Acad. Sci. 47,* 1-55.

Zamecnik, P. C., and E. B. Keller. 1954. Relation between phosphate energy donors and incorporation of labeled amino acids into proteins. *J. Biol. Chem. 209,* 337-354.

III. PLANT CILIA AND ASSOCIATED ORGANELLES

BY I. MANTON [1]

THE work done in Leeds on plant cilia since 1949 has been summarized twice before (Manton, 1952, 1954), and it will therefore be appropriate to begin at the point where the last summary ended, namely, with our reconstruction of the internal anatomy of the cilia of the moss *Sphagnum*, Fig. 1 (after Manton and Clarke, 1952). Before discussing this diagram, however, it will perhaps be appropriate to outline the

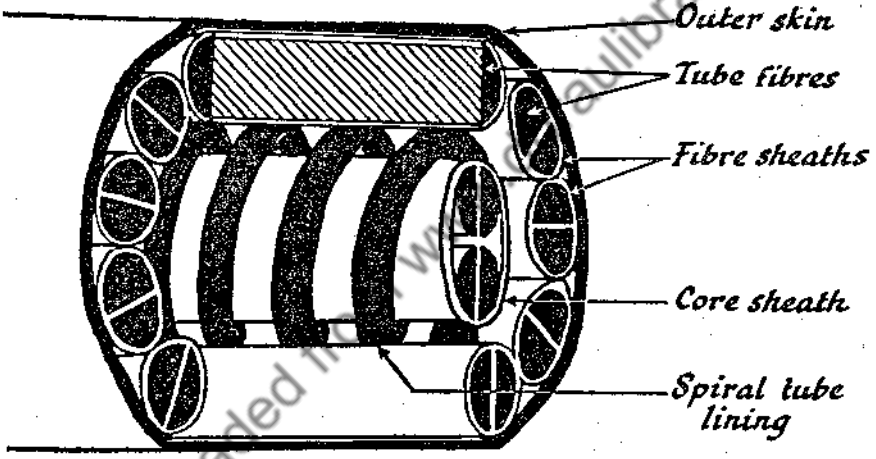


Fig. 1. Diagrammatic reconstruction (after Manton and Clarke, 1952) of the micro-anatomy of a plant cilium based on the evidence from the moss *Sphagnum* but thought to be of general applicability.

general scope of the research program, which began with ultraviolet microscopy and led on into electron microscopy and which is still in progress. In this way, a newcomer to these topics may be able to put the present position into its proper setting.

Our interest in cilia began, as is well-known, with a chance observation made in 1949 with my ultraviolet microscope, then newly designed and delivered to Leeds—that the cilia of a fern were many-stranded. The strands were not fully resolvable, but they were illustrated in the frontispiece to a book on fern cytology which I published in 1950 and

[1] Botany Department, University of Leeds, England.

in a small paper (Manton, 1950b) published to amplify this frontispiece. The number of strands was estimated as "of the order of 10," an estimate which proved to be surprisingly accurate since we all now know that the number in plants and animals generally is exactly 11. As soon as the paper was published, I was assailed by a flood of correspondence pointing out that many light microscopists had made comparable observations before. There is, indeed, considerable literature on the subject, most of which is summarized in Manton and Clarke (1951a). Few of these earlier workers, however, had ventured on an exact numerical estimate. In a personal communication, J. R. G. Bradfield recently brought to my attention the fact that one zoologist (Ballowitz) made the exact count of 11 strands in 1888, though only on a single macerated sperm tail of the Chaffinch for which the average estimates were 7-10; this achievement for its date was a remarkable one.

With the arrival of the Philips' electron microscope into our own Department in 1950, we set out first to examine more exactly the fibrillar components of cilia. As working botanists we had considerable experience in methods of obtaining ciliated cells from the various plant groups. This experience enabled us to survey the more important known types comparatively rapidly. In so doing we obtained some surprising observations on external morphology, some of which will be discussed below, and we showed in group after group—with such completeness as to admit of no exceptions in plants—that the number of component fibrils of even the most diverse ciliary types was invariably exactly 11. Earlier electron microscopists had, of course, begun to make comparable observations on animals, notably, Jakus and Hall (1946) on the cilia of *Paramoecium* and Grigg and Hodge (1949) on the sperm tails of the fowl. We have naturally never claimed to have been the first to discover the 11 strands. On the other hand, we do seem to have been among the first investigators who deliberately attempted to establish the facts on a sufficient scale to generalize from them. It was therefore no accident that by the time Dr. D. Fawcett cut the first thin sections of cilia in animals (Fawcett and Porter, 1954), we had already, without the aid of sections, arrived at the conclusions assembled in Fig. 1. I can say without risk of exaggeration, and I hope that I shall be allowed to print the comment, that the discovery of the close agreement between our reconstruction and Fawcett's sections was one of the most delightful and exciting experiences of my life, and that it contributed more than a little to keeping me alive during a serious illness which at that point had interrupted work for several months. As soon as possible after

convalescence, I came to America to learn Fawcett's (i.e. Porter's) technique, a task completed in the spring of 1954 in Dr. K. R. Porter's Laboratory at the Rockefeller institute. This led to the even more delightful discovery that Fawcett and I were probably both wrong in one rather crucial detail of interpretation to which I will return below. In the meantime Challice (1954) and Bradfield (1954) had both published sections of animal sperm tails, thereby completing the demonstration of the extraordinary uniformity of internal structure among cilia and flagella throughout the plant and animal kingdoms above the level of bacteria.

With the advent of thin sectioning on a routine basis a new field of enquiry has opened up which is still only in the earliest stages. Our first objective has been to confirm the details of our previous work (Fig. 1) and to extend or correct them if possible. Secondly, the access to intracellular structures which sectioning permits has enabled us to begin to explore the way in which cilia in plants are attached to the cell. We have not progressed nearly as far in this as Fawcett and Porter (1954) were able to do in animals, partly because in plants we have no ciliated epithelium but usually have to examine our cilia singly or borne in pairs or in other simple arrangements on separate cells. Some preliminary results will, however, be discussed below.

Returning now to Fig. 1, the detailed components are all labeled, and since the evidence for them was discussed in detail in 1954, it will not be recapitulated here. It is sufficient to say that we had direct evidence for the total number of strands and for their arrangement in a ring of 9 around a central pair (cf. Plate I), but not for the circular cross section of the whole organ which was inferred as probable. In the same way we had evidence for the longitudinal split down each of the 9 peripheral fibrils and some, though less extensive, evidence for a similar split down each of the central 2; we had, however, no means of knowing the plane in which the split might lie, and this part of the diagram was therefore also hypothetical. Of the other components we had direct evidence for the ciliary skin and for the fibril sheaths including the common sheath round the central pair. We had also direct evidence for a segmentally arranged cross banding material inside and attached to the tube of 9 strands and touching, but not attached to, the central core. This component is indicated in black in the diagram, but the exact configuration as a spiral rather than as some less regularly banded shape was hypothetical. This component is difficult to detect since it is by far the most labile and is only occasionally preserved in dried material and never

in embedded material. We have, however, seen it sufficiently often in mosses and algae to make a reasonable claim that it is of regular occurrence.

Fawcett and Porter's sections of cilia on two molluscs, two amphibia, and two mammals (1954) agreed exactly with the following details of our diagram: (1) the circular cross section, except when flattening by the knife had produced deformation; (2) the 9 plus 2 arrangement of fibrils within the ciliary skin; and (3), in the favorable case of the frog, the radial direction of the split down each of the peripheral strands. Similar observations have since been made in sections of plant cilia (cf. Manton, 1955, and Plate V of this paper) and on the tails of Echinoderm spermatozoa (Afzelius, 1955). The essential correctness of the *Sphagnum* diagram is therefore substantially confirmed.

Fawcett and Porter were also able to observe that when cilia are arranged in rows as in the gill epithelium of *Mya*, the line joining the central strands in any one cilium is at right angles to the direction of the row. In plants we have found a similar arrangement where cilia arise in pairs, as in the green algae with equal cilia (see Plate V, 3) or even when the pairs are unequal as in the flagellate *Synura* (Manton, 1955). The orientation of cilia on a surface is thus by no means at random, and it seemed highly probable that the facts of dorsiventrality and bilateral symmetry conferred by the 9 plus 2 arrangement might be of major functional significance for the mechanism of their movement.

But at this point there was uncertainty as to the interpretation of the two essential planes. Both Fawcett and I had at first assumed that the line joining the two central strands would mark the plane of bilateral symmetry. It should, however, have been obvious that the median line could equally well be at right angles to this and pass not through but between the two central strands. In a section of a cilium without external appendages or other distinguishing marks it is impossible to decide between these two alternatives by mere inspection, and this is the point at which the new work in plants may be said to begin. Before discussing it, however, it is necessary to give a brief digression on the external appendages of plant cilia since without these the problem could scarcely have been solved on botanical material.

In plants the cilia of certain groups are as devoid of appendages as are those in the majority of animals: these groups include all the land plants (Bryophyta, Pteridophyta, and probably Gymnosperms), green algae, and certain fungi and flagellates. In other groups, with greater or less frequency, we find fins, hairs, or spines distributed upon the cilia

in various arrangements. Fins are uncommon but we have previously shown what we believe to be a good example in the hind flagellum of the second stage zoospores of the fungus *Saprolegnia* (Manton, Clarke, and Greenwood, 1951), though this has not yet been confirmed by means of a section. Hairs are highly characteristic of several important algal and fungal groups and they are of the greatest interest to botanists for an entirely different purpose, namely, the tracing of phylogeny. Thus a single row of hairs is characteristic of the flagellates associated with *Euglena.* Two rows of hairs or of tufts of hairs occur on the front flagellum in brown algae, yellow green algae (formerly called Heterokontae) and certain groups of fungi and flagellates which for that reason are thought to be related. Some recently investigated examples are illustrated in Plates II to IV, the flagellate *Synura* (Plate IV) being given in the fullest detail. Spines are the rarest type of appendage. We have so far found them in only three cases, all among the spermatozoids of brown algae. One of the most spectacular of these is the British seaweed *Himanthalia lorea* (Manton, Clarke, and Greenwood, 1953) in which the spermatozoid carries an immense spine over a micron long near, but not at, the distal end of its front flagellum (Plate III, 4). This flagellum also carries the two rows of lateral hairs customary in this group which may be seen at the sides of the dismembered specimen in Plate III, 5. This figure also shows clearly that the spine is attached to one particular fibril of the peripheral series though it is not possible to determine exactly which fibril this is. A similar spine has recently been detected in the New Zealand fucoid *Xiphophora chondrophylla,* from which, it may be said in passing, a relationship to the European genus *Himanthalia* may perhaps be inferred in a way which would have been difficult to demonstrate otherwise. Since this work has not yet been published, a visual light view of the spermatozoid (Plate III, 2) has been reproduced beside the electron microscope view of parts of two front flagella carrying the single spine and traces of the lateral hairs (after Flint, Manton, and Clarke, unpublished). Finally, there is *Dictyota* with a row of spines (Plate II) placed in a median position between the two lateral rows of hairs. This peculiarity is so important that it must detain us a little longer.

Dictyota was the context in which we first explicitly discussed the question of symmetry in cilia (Manton, Clarke, and Greenwood, 1953). The existence of bilateral symmetry as inherent in the numerical relations of the 9 plus 2 fibers had been clear to us from the beginning. But without the evidence from *Dictyota,* we would have had no means of demonstrating the existence of a definite morphological differentiation

in the dorsiventral direction, although this in a less conspicuous way is also inherent in the fibre numbers. When a cilium has either no appendages or only one row, there is no possibility of distinguishing with certainty between the various planes. With two rows there are still two alternatives, namely, that the appendages may either lie in or be symmetrically disposed at right angles to the plane of symmetry. With three, however, the planes can be precisely defined even without the aid of a section, at least as far as the peripheral fibres are concerned. As may be seen from the dismembered specimen of Plate II, the median fibril of *Dictyota* is carrying the whole row of spines in a serial array, and if the position of the spines on the intact cilium is then determined, it is found to lie outwards relative to the body (Plate II). It was thus clear, in the first place, that the plane of bilateral symmetry in the cilia of *Dictyota* is at right angles to the surface of the body along which the cilium lies; secondly, that the median fibril is outwards; and, thirdly, that the two rows of hairs are lateral as they had long been thought to be.

Had we been able at that time to cut a transverse section through the critical region of *Dictyota,* the remaining details would have been settled forthwith. We were not in this position, and our results were expressed diagrammatically in the simplified form of Fig. 2A. This diagram is correct where it is based on direct observation, i.e. in the position of the appendages relative to the fibrils and cell body. It is not correct, however, where it is based on inference, i.e. in the position assigned to the central fibrils. For this I must absolve my colleagues and collaborators and take personal responsibility, tempered perhaps by the fact that I was in the hospital at that time. The diagram is a modification of a simplified version of the geometry of the 11 strands published in Manton and Clarke (1952) which we now know to have been in some respects misleading. The diagram which we ought to have published on the evidence available in 1953 is shown in Fig. 2B.

In order to distinguish between the two alternative positions of the central fibrils as expressed in Fig. 2B, it is necessary to obtain a transverse section through a cilium in which the central strands can be visibly related either to an externally marked median fibril, as in *Dictyota,* or to the two externally marked lateral lines of hairs. Material for the former has not yet been available since *Dictyota* itself only liberates its motile cells at the spring tides in August and September and it failed to do so in the exceptionally unfavorable weather conditions of 1954. We have therefore concentrated attention on the two lateral lines and,

after a few false starts, we think we have succeeded. One of the false starts is represented in Plate IV, 2, included to show one of the reasons why this observation is not as easy to make as it may seem to be. The flagellate *Synura* possesses the necessary lines of hairs but it also possesses an exceptionally wide superficial sheath within which nothing is preserved after embedding except the ciliary fibrils themselves. These commonly lie excentrically within the sheath after sectioning but their connection if any with the external hairs is then impossible to deter-

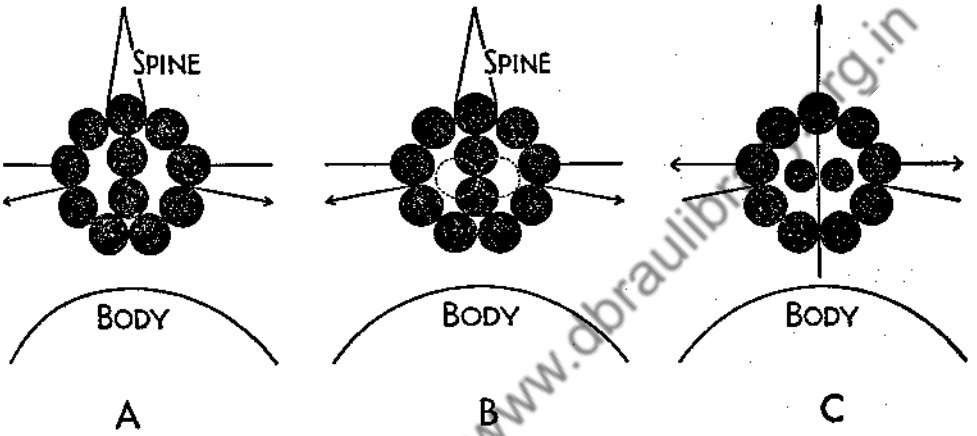


Fig. 2. Some possible arrangements of the 11 strands of a plant cilium in relation to external appendages; the pairs of lateral lines in each case indicate alternative positions for the lateral rows of hairs, the positions most probable at the date of publication distinguished by arrow heads. A. Reconstruction of *Dictyota* from morphological evidence only (after Manton, Clarke, and Greenwood, 1953). B. A more correct version of (A) allowing for two alternative positions of the central strands. C. Arrangement of the front flagellum of *Fucus* as seen in section (plate V, 4); the long arrow marks the plane of bilateral symmetry.

mine. This difficulty is not present, however, in *Fucus* in which the skin is close-fitting though liable to disappear. The main difficulty here is that there is no correspondence in level between the tufts of hairs emerging on the two sides, and some obliquity of the section is inevitable if both are to be traceable simultaneously. This does not necessarily exclude successful resolution of the central fibrils, and a section containing all the essential information is reproduced in Plate V, 4. This section shows unmistakably that the lateral hair lines and the two central strands are roughly in line with one another and that the median plane (marked by the arrow) must therefore pass between but not through the two central fibrils. The relation of this section to the front end of the body of the cell also shows that the median fibril of the pe-

ripheral ring is outwards as in *Dictyota*. These facts are summed up in Fig. 2C, and the only details still remaining in slight uncertainty concern the exact position of the hairs. If the median fibril is numbered 0 and the others 1, 2, 3, 4, on either side, there is no doubt that fibril 2 is closely associated with the origin of the hairs both in *Dictyota* and in *Fucus*. In *Dictyota* we were, however, uncertain whether the hairs were borne actually on fibril 2 or between fibrils 2 and 3. At that time we were inclined to think the latter the more probable, hence the arrowhead on the lines depicting this position in Fig. 2A. The evidence from *Fucus* is now more in favor of the former (Fig. 2C).

With the knowledge that the plane containing the two central fibres in a cilium is at right angles to the plane of symmetry of the whole organ, it is possible to extend these observations to other types of cilia and flagella. The smooth hind flagellum of the *Fucus* spermatozoid may be quoted as an example. This is held in a fixed position where it passes over the eyespot and in sections of this region it is again found that the plane of symmetry is perpendicular to the body and the median fibril probably outwards. These findings suggest that though here the flagellum is not externally differentiated dorsiventrally, it is not a matter of indifference which way up it is placed. All this endorses very strongly our previous view (Manton, Clarke, and Greenwood, 1953) that the attributes of bilateral symmetry and dorsiventrality are likely to be of primary importance for the functioning of these organs and that they are intimately concerned with the numerical stability of the 9 plus 2 arrangement.

The remaining new developments must be passed over more rapidly. The basal parts of plant cilia have been examined externally in a few cases, notably in the fungi *Olpidium* and *Allomyces* (Manton, Clarke, Greenwood, and Flint, 1952); in greater detail in the brown alga *Himanthalia* (Manton, Clarke, and Greenwood, 1953); and in the green algae *Draparnaldia* and *Chaetomorpha* (Manton, Clarke, and Greenwood, 1955). Two of our figures for *Draparnaldia* are reproduced from the latter paper (Plate VIII) to show the basal bodies of the cilia, the fibrous connections uniting them, and the fibrillar composition of the "roots" which arise between them. In sections we have not yet reached the completeness attained by Fawcett and Porter for animal cells though a beginning has been made. We have sections through the basal bodies of the flagellate *Synura* (Plate VII), of the zoospores of the brown alga *Scitosiphon* (Plate VI, 2), the spermatozoids of *Fucus*, and the coenozoospores of the filamentous "green" alga *Vaucheria* (Plate VI,

1). In all these the basal bodies of the cilia are hollow and each is separated from the cilium itself by an opaque diaphragm placed exactly at the level of the cell surface. The walls of the basal bodies vary in shape and in thickness but they all show fibrous markings which almost certainly bear a precise numerical relation to the fibres of the peripheral ring. The central fibres do not penetrate through the diaphragm but we are less certain than Fawcett and Porter appeared to be that these fibres end above the diaphragm. We believe that in most of our material these fibres end in the diaphragm though the evidence is not yet perfect.

Of greater intrinsic interest at the present stage of the enquiry are the relations between the ciliary bases and other cell organs. In *Scitosiphon* (Plate VI, 2) and in *Vaucheria* (Plate VI, 1) the bases are almost touching the nucleus which is drawn out into a peaked extremity towards them (most clearly shown in Plate VI, 2). There is no reason to suppose that any appendages from the cilia penetrate into these nuclei though they are undoubtedly attached to them. In *Synura*, in contrast, ciliary bases are relatively remote from the nucleus but they are visibly attached to it by means of obliquely striated "roots" which can be seen descending from the basal bodies towards the nucleus in both the figures on Plate VII (marked by short arrows). Their number is not less than two per cilium at their point of origin though it may be more and it is certainly more after contact with the nuclear surface has been made. At this point (Plate VII, 1) a root bends sharply and branches though it may also perhaps branch at earlier levels. The ultimate branchlets pass down both sides of the nuclear surface closely pressed to it. In addition there are other non-striated roots which pass outwards and upwards from the ciliary bases of *Synura* to an ultimate destination which has not yet been traced.

These observations lead on to other types of problem. The relation between the hind flagellum and the eyespot is certainly a close one in several of our forms, e.g. *Fucus* and *Scitosiphon* (Plate VI, 2) though the details differ. The relation to mitochondria is also engaging our attention though again the details differ; in most cases we find a distinct accumulation of these organelles close to the ciliary bases though in the special case of *Vaucheria*, which is peculiar in many ways, there is an accumulation of mitochondria round the nuclei to which the cilia are attached rather than explicitly round the cilia. Lastly, there is the very peculiar organ visible on one side of the cilia in both specimens of *Synura* included on Plate VII (marked by the long arrow), composed

of a stack of oblique and somewhat contorted lamellae apparently joined together at their edges and always to be found in this particular place. It seems to resemble an organ described in *Euglena* by Wolken and Palade (1953) as a part of the endoplasmic reticulum. Whether this is sufficient designation we are not prepared to say, but its close association with the cilia suggests that it may be metabolically concerned with them.

Observations such as these are leading us rapidly away from the restricted topic of cilia to widely different problems of the structure and function of the different parts of cells. This is therefore perhaps the moment to conclude this survey and to sum up.

The use of sections has added clarity and completeness to our previous account of plant cilia though some components can still only be studied effectively in whole mounts. Our diagram (Fig. 1) has been substantially confirmed and the close similarity between plants and animals has been amply demonstrated not only for cilia in themselves but also with respect to their basal parts. With regard to the cilia themselves the most important new observation is that establishing the plane of bilateral symmetry as passing between, but not through, the two central strands. Finally, the close relation of the cilia to the cell nucleus in plants together with the presence of other unexplained organs in their immediate vicinity are raising problems which so far do not seem to have been encountered to the same extent in animals and on which enquiry is continuing.

BIBLIOGRAPHY

Afzelius, B. A. 1955. The fine structure of the sea urchin spermatozoa as revealed by the electron microscope. *Z. Zellforsch. 42,* 134-148.

Ballowitz, E. 1888. Untersuchungen über die Struktur der Spermatozoën, zugleich ein Beitrag zur Lehre vom feinerem Bau der contraktilen Elemente. *Arch. mikr. Anat. 32,* 401-473.

Bradfield, J. R. G. 1953. New features of protoplasmic structure observed in recent electron microscope studies. *Quart. J. Micr. Sci. 94,* 351-367.

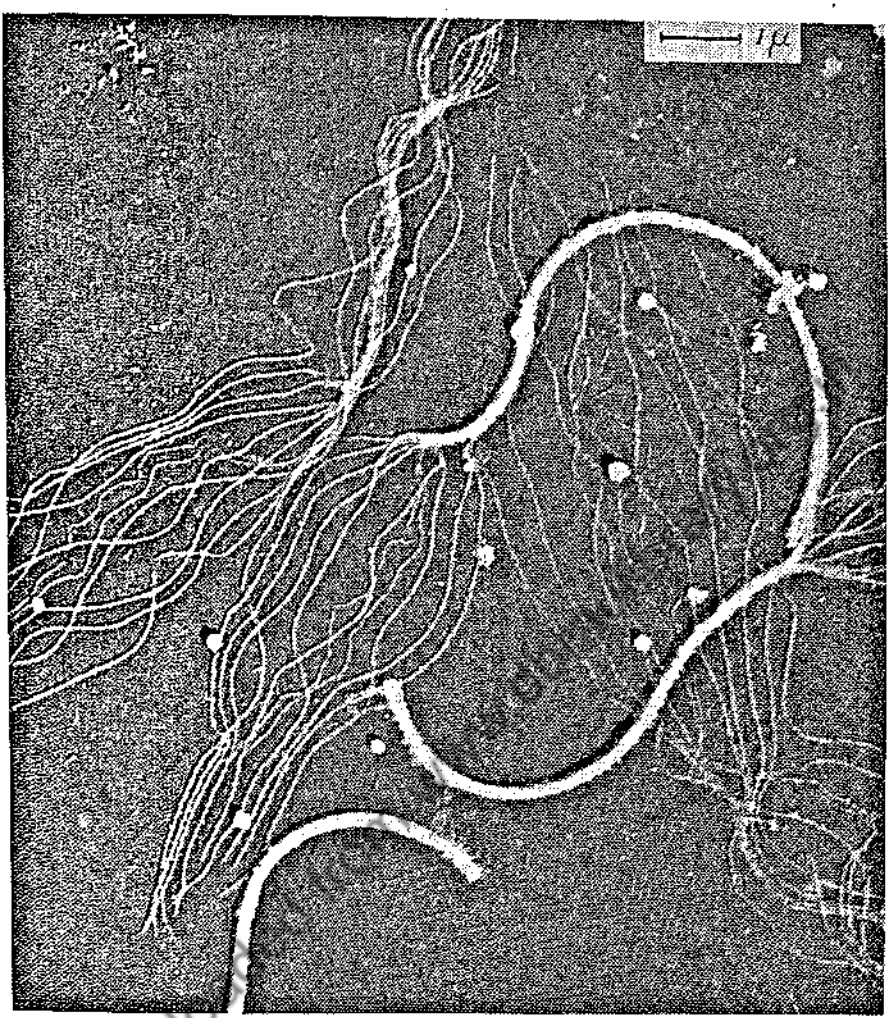
Challice, C. E. 1953. Electron microscope studies of spermiogenesis in some rodents. *J. Roy. Micr. Soc. Ser. 3, Pt. 3, 73,* 115-127.

Fawcett, D. W., and K. R. Porter. 1952. A study of the fine structure of ciliated epithelial cells with the electron microscope. *Anat. Rec. 113,* 539 (abstr.).

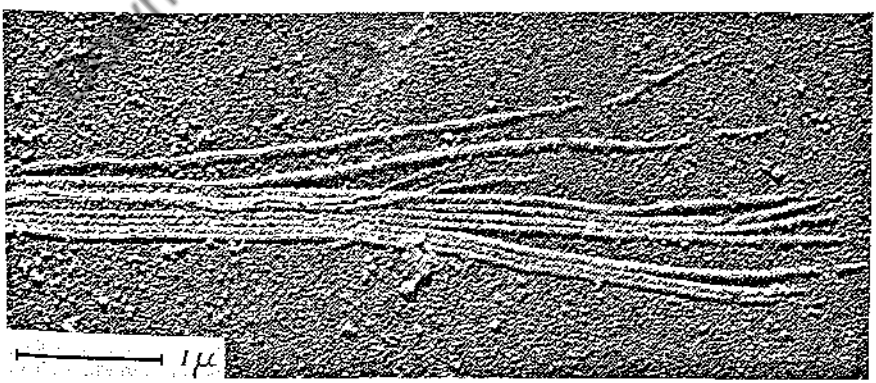
Fawcett, D. W., and K. R. Porter. 1954. A study of the fine structure of ciliated epithelia. *J. Morph. 94,* 221-282.

Grigg, G. W., and A. J. Hodge. 1949. Electron microscopic studies of spermatozoa. I. The morphology of the spermatozoon of the common domes-

tic fowl (*Gallus domesticus*). *Aust. J. Sci. Res. Ser. B, 2,* 271-286.
Manton, I. 1950a. Problems of cytology and evolution in the Pteridophyta. Cambridge.
Manton, I. 1950b. Demonstration of compound cilia in a fern spermatozoid by means of the ultra-violet microscope. *J. Exp. Bot. 1,* 69-70.
Manton, I. 1952. The fine structure of plant cilia. *Symposia Soc. Exp. Biol. VI,* 306-319.
Manton, I. 1954. New work on the internal structure of plant cilia. *Proc. 1st Internat. Con. Electron Microscopy, London.*
Manton, I. 1955. Observations with the electron microscope on *Synura caroliniana. Proc. Leeds Phil. Soc. 6,* 306-316.
Manton, I., and B. Clarke. 1951a. Demonstration of compound cilia in a fern spermatozoid with the electron microscope. *J. Exp. Bot. 2,* 125-128.
Manton, I., and B. Clarke. 1951b. Electron microscope observations on the zoospores of *Pylaiella* and *Laminaria. J. Exp. Bot. 2,* 242-246.
Manton, I., and B. Clarke. 1952. An electron miscroscope study of the spermatozoid of *Sphagnum. J. Exp. Bot. 3,* 265-275.
Manton, I., B. Clarke, and A. D. Greenwood. 1951. Observations with the electron microscope on a species of *Saprolegnia* I. *J. Exp. Bot. 2,* 321-331.
Manton, I., B. Clarke, and A. D. Greenwood. 1953. Further observations with the electron microscope on spermatozoids in the brown algae. *J. Exp.. Bot. 4,* 319-329.
Manton, I., B. Clarke, A. D. Greenwood, and E. A. Flint. 1952. Further observations on the structure of plant cilia, by a combination of visual and electron microscope. *J. Exp. Bot. 3,* 204-215.
Manton, I., B. Clarke, and A. D. Greenwood. 1955. Observations with the electron microscope on biciliate and quadriciliate zoospores in green algae. *J. Exp. Bot. 6,* 126-128.
Wolken, J. J., and G. E. Palade. 1953. An electron microscope study of two flagellates. Chloroplast structure and variation. *Ann. N.Y. Acad. Sci. 56,* 873-889.



1



2

PLATE I. 1. Detached cilia from the spermatozoid of the Male Fern (*Dryopteris Filix-mas*) showing fibrillar disintegration into 9 coarser strands and 2 central finer strands in each cilium. Electron micrograph M.16.2, negative print, uranium shadowing, 60 kV, ×10,000. 2. Less fully dismembered cilium from the zoospore of the green alga *Draparnaldia* sp. Electron micrograph M.81.2, negative print, gold palladium shadowing, 60 kV, ×20,000. (After Manton, Clarke, and Greenwood, 1955.)

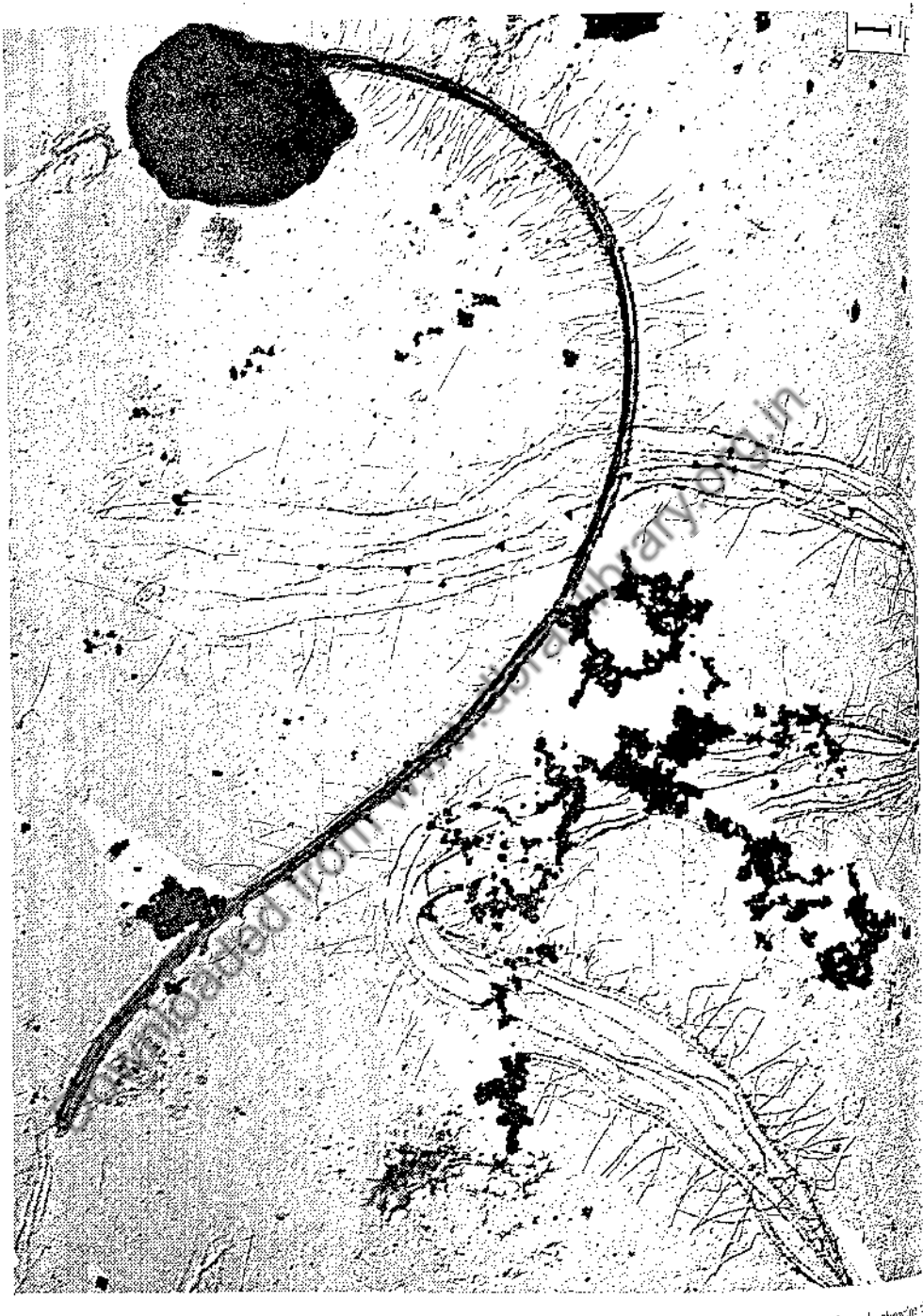
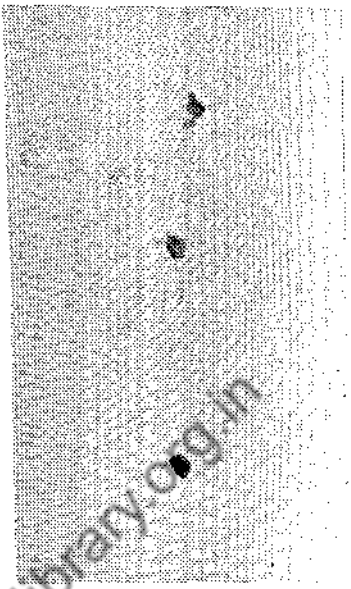
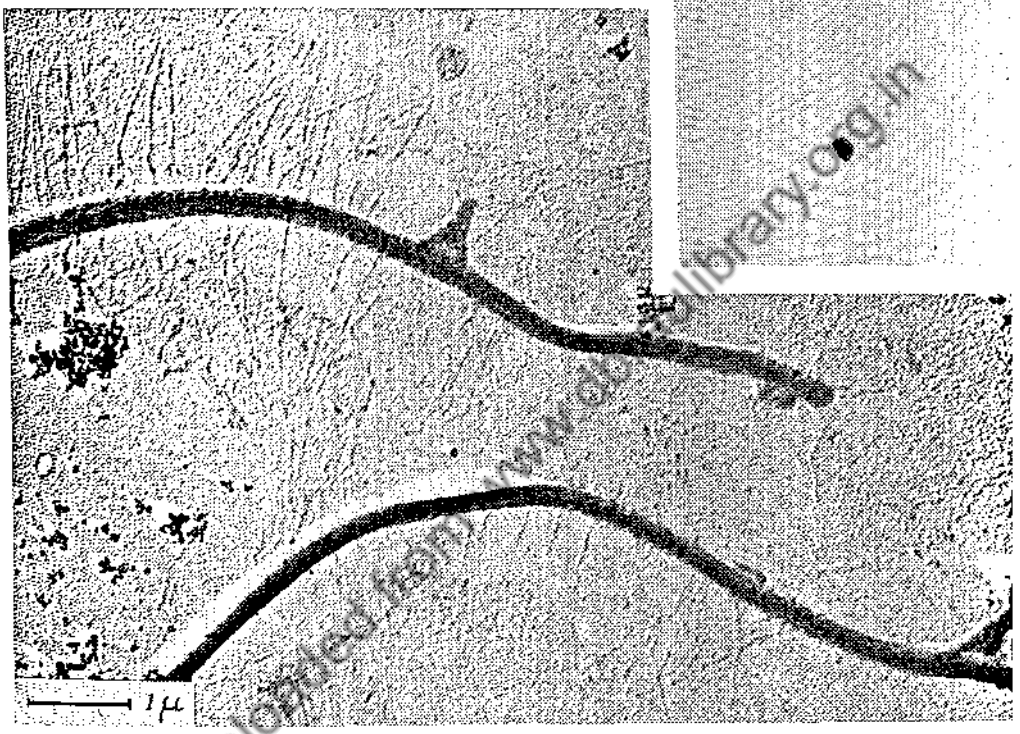


PLATE II. Two spermatozoids of the brown alga *Dictyota dichotoma*, one intact and the other dismembered, showing lateral hairs and the median row of spines. Electron micrograph M.93.27, gold-palladium shadowing, 60 kV, X (After Manton, Clarke, and Greenwood, 1953.)

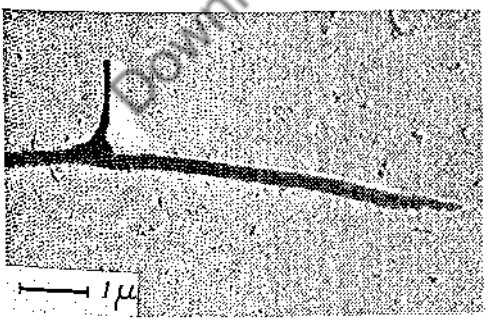
1



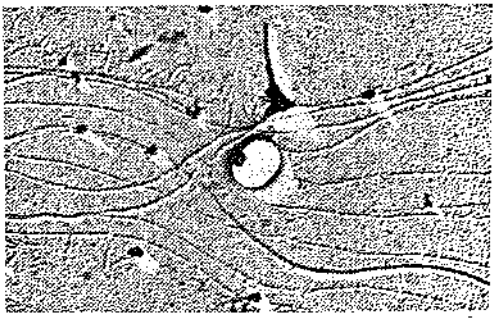
2



3



4



5

PLATE III. 1. Part of the front flagellum of the zoospore of the brown alga *Pylaiella litoralis* to show the bases of the lateral hairs as they emerge in tufts from the axis. Electron micrograph M.30.15, uranium shadowing, 40 kV, ×40,000. (After Manton and Clarke, 1951.) 2. Photograph with the light microscope of the cilia on three spermatozoids of the brown alga *Xiphophora chondrophylla* dried on glass in New Zealand and photographed without a coverslip. ×1,000. (After Flint, Manton, and Clarke, unpublished.) 3. Parts of the front flagella of two similar spermatozoids stripped from glass and remounted for the electron microscope showing traces of lateral hairs and a spine-like organ. Electron micrograph M.175.6, gold-palladium shadowing, 60 kV, ×15,000. (After Flint, Manton, and Clarke, unpublished.) 4. Tip of the front flagellum of a spermatozoid of the brown alga *Himanthalia lorea* showing the spine. Electron micrograph M.96.29, gold-palladium shadowing, 60 kV, ×10,000. (After Manton, Clarke, and Greenwood, 1953.) 5. Comparable to the last but flagellum disintegrated; lateral hairs, the II strands, and the attachment of spine are visible. Electron micrograph M.92.7, ×10,000. (After Manton, Clarke, and Greenwood, 1953.)

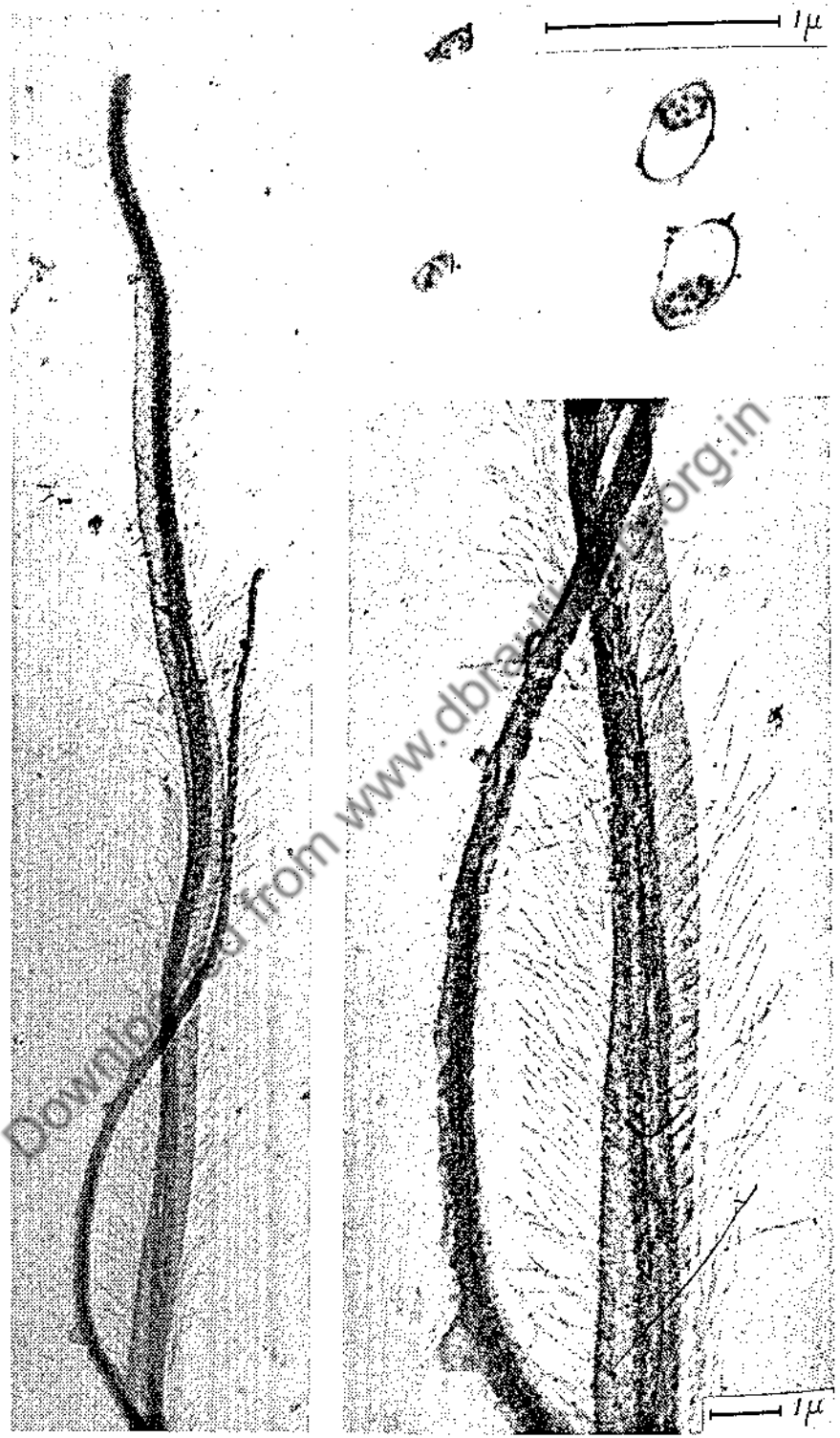
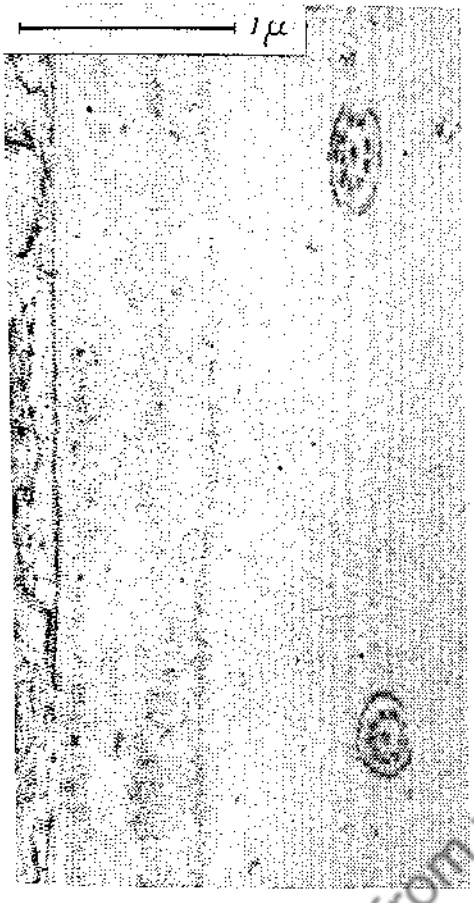
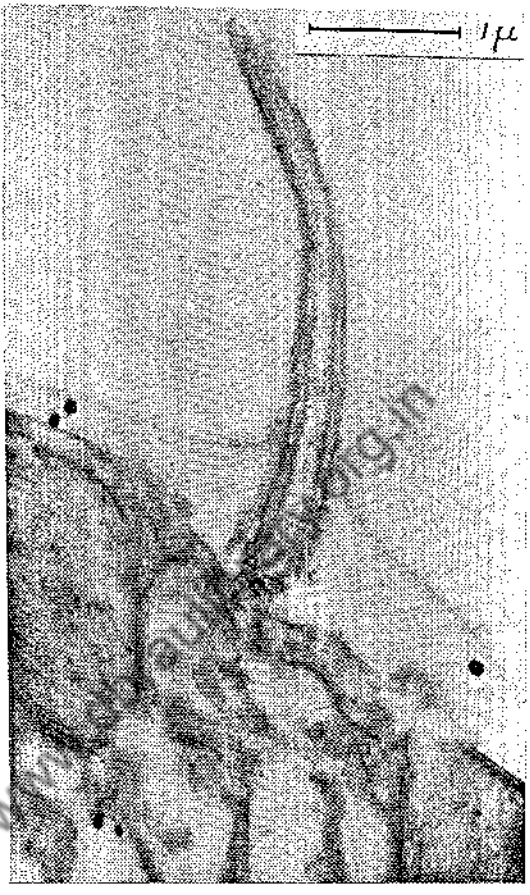


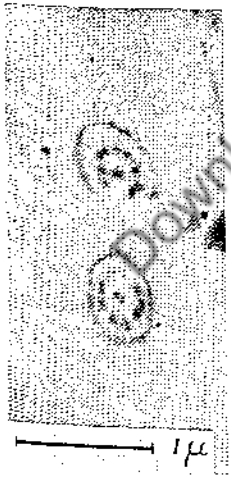
PLATE IV. 1. Pair of cilia from a cell of the colonial flagellate *Synura caroliniana* showing their unequal size. Electron micrograph M.137.20, 60 kV, ×6,000. (After Manton, 1956.) 2. Section of two parts of cilia of *Synura caroliniana*, only the larger ones showing internal structure. Micrograph 2M.1 taken on the RCA microscope at the Rockefeller Institute, New York, ×33,000. (After Manton, 1956.) 3. Part of the base of (1) more highly magnified to show the lateral hairs and loose sheath on the large flagellum. Electron micrograph M.137.21, ×10,000.



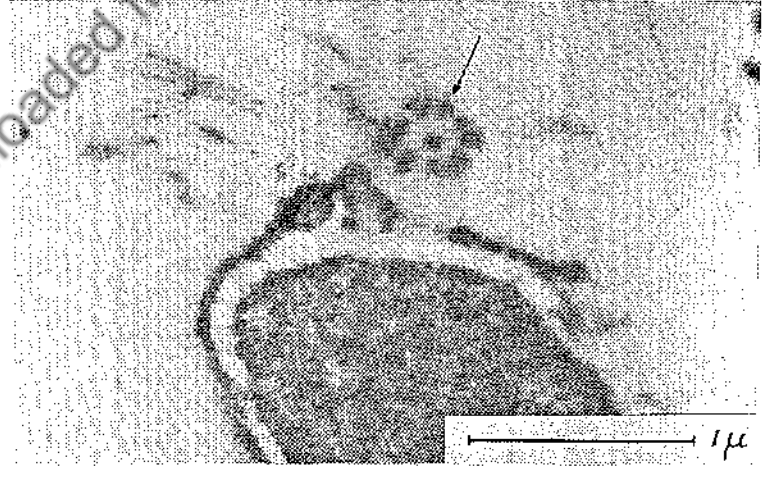
1



2

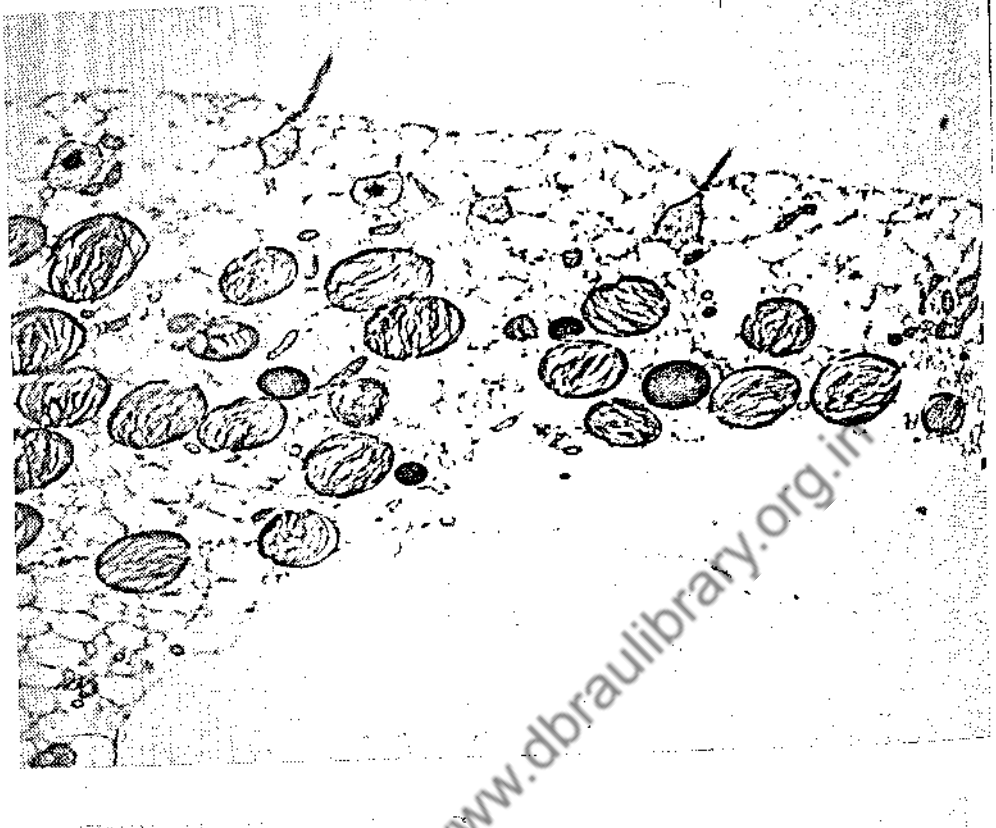


3

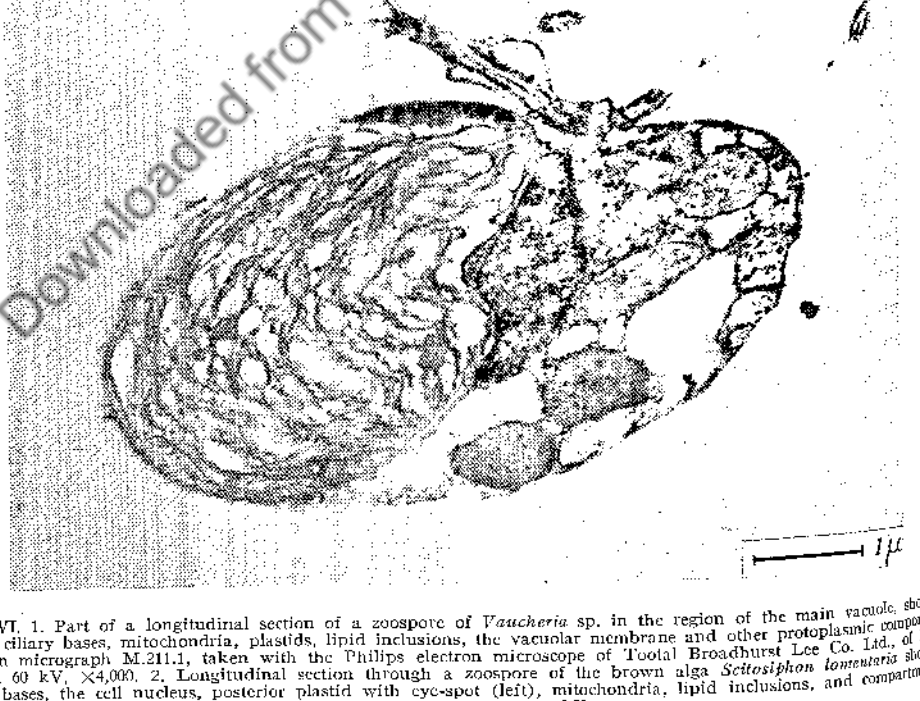


4

PLATE V. 1. Section of a pair of cilia near the edge of a colony of the green alga *Eudorina*. Electron micrograph R10.19, taken on the Philips microscope at the Rockefeller Institute, ×30,000. 2. Longitudinal section of a cilium on the colonial green alga *Pandorina*, taken on the Philips microscope at the Rockefeller Institute, ×20,000. 3. Transverse section of a pair of cilia of *Pandorina* showing the parallel symmetry of the two. Electron micrograph M.133.7, 60 kV, ×20,000. 4. Slightly oblique transverse section near the base of the front flagellum of the spermatozoid of *Fucus serratus* showing the positions of the internal fibrils in relation to the lateral hairs and front end of the body of the cell; the arrow marks the plane of bilateral symmetry of the cilium. Electron micrograph M.213.6, taken with the Philips microscope of Tootal Broadhurst Lee Co. Ltd., of Manchester, ×30,000.

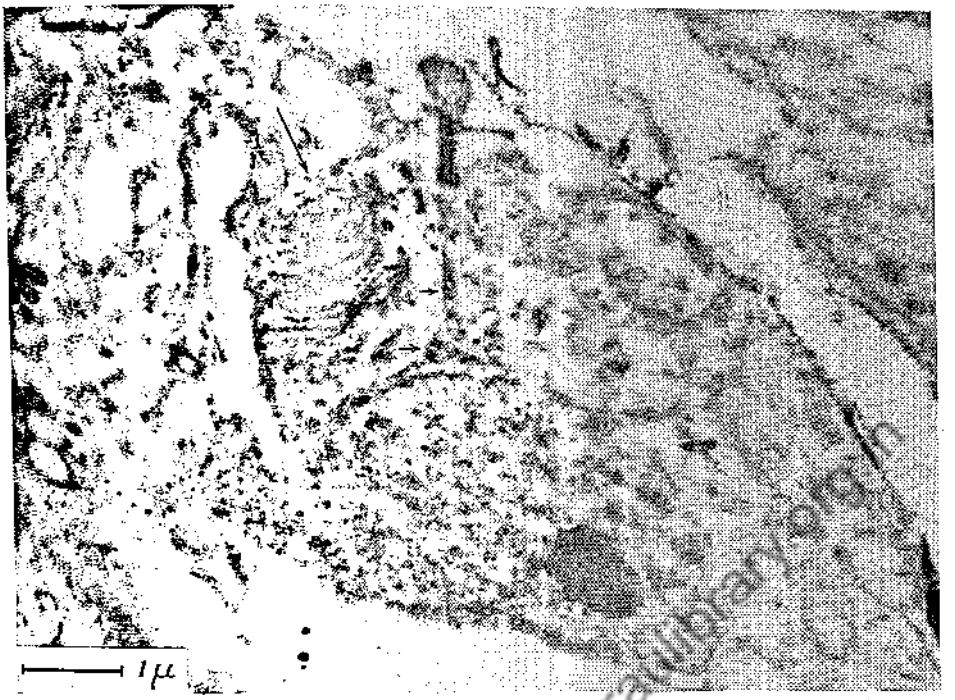


1

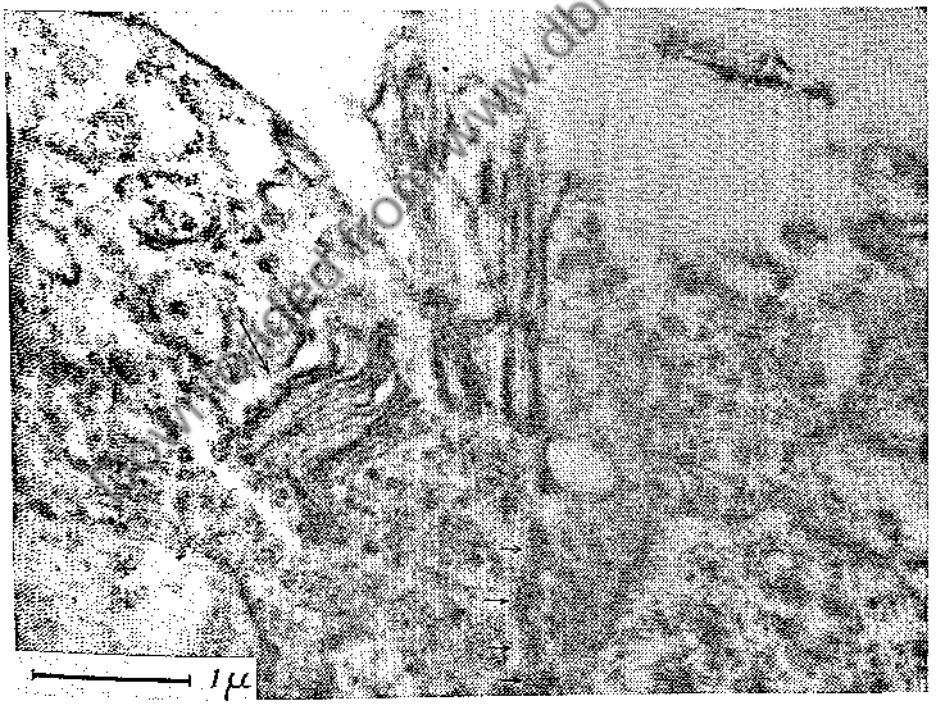


2

PLATE VI. 1. Part of a longitudinal section of a zoospore of *Vaucheria* sp. in the region of the main vacuole, show nuclei, ciliary bases, mitochondria, plastids, lipid inclusions, the vacuolar membrane and other protoplasmic compone Electron micrograph M.211.1, taken with the Philips electron microscope of Tootal Broadhurst Lee Co. Ltd., of M chester, 60 kV, ×4,000. 2. Longitudinal section through a zoospore of the brown alga *Scytosiphon lomentaria* show ciliary bases, the cell nucleus, posterior plastid with eye-spot (left), mitochondria, lipid inclusions, and compartmer protoplasm at anterior end (right). Electron micrograph M.206.20, 60 kV.

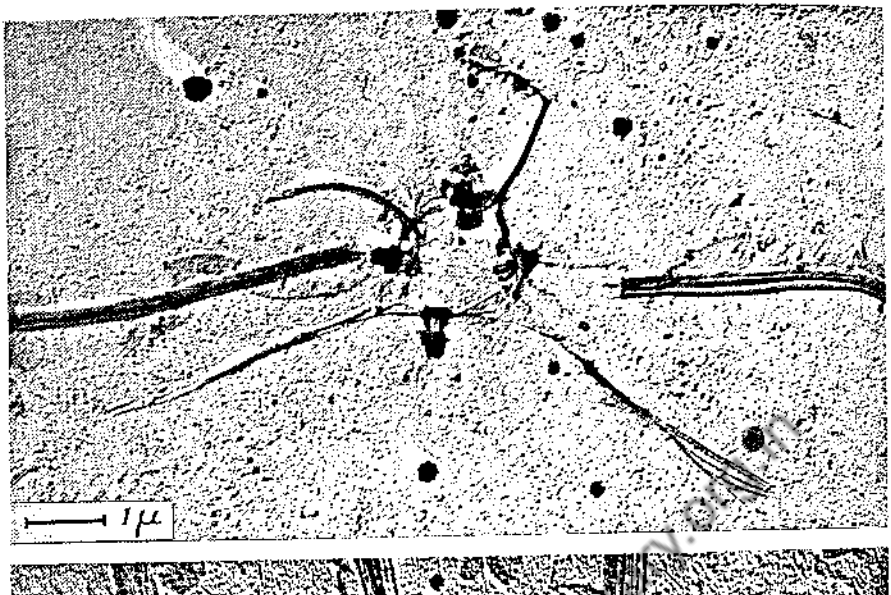


1

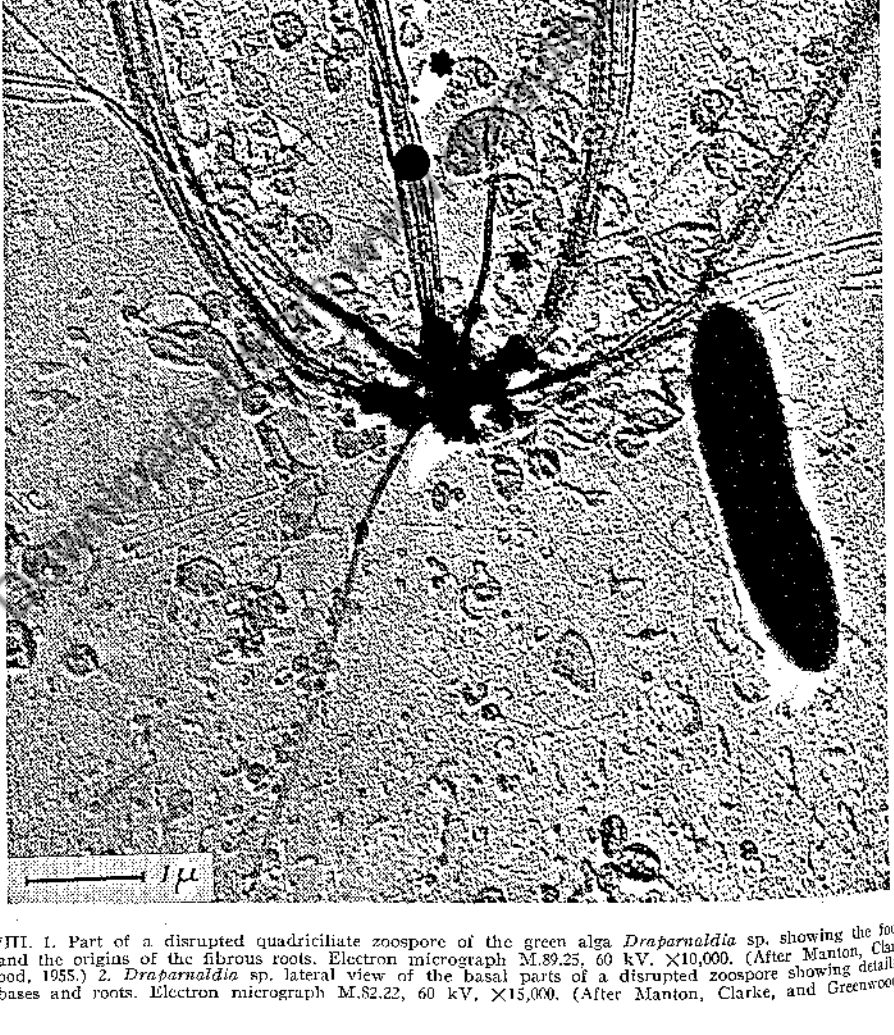


2

Plate VII. 1. Part of a longitudinal section near the distal end of a cell of *Synura caroliniana* showing the cell nucleus with contained nucleolus, part of the base of a cilium, some cross banded "roots" (short arrows) descending from the ciliary base onto the surface of the nucleus and a peculiar stack of lamellae (long arrow) placed near them. Electron micrograph M.169.29, ×15,000. (After Manton, 1956.) 2. Part of a similar section, more highly magnified, showing the bases of two cilia. Electron micrograph M.170.17, ×22,500.



1



2

PLATE VIII. 1. Part of a disrupted quadriciliate zoospore of the green alga *Draparnaldia* sp. showing the four ba
bodies and the origins of the fibrous roots. Electron micrograph M.89.25, 60 kV. ×10,000. (After Manton, Clarke,
Greenwood, 1955.) 2. *Draparnaldia* sp. lateral view of the basal parts of a disrupted zoospore showing details of
ciliary bases and roots. Electron micrograph M.82.22, 60 kV. ×15,000. (After Manton, Clarke, and Greenwood, 19

IV. PATTERN AND SUBSTANCE IN STENTOR

BY VANCE TARTAR [1]

The ciliate protozoan *Stentor coeruleus* presents at least two notable advantages to the student of morphogenesis. First, there is in this unicellular organism a visible cytoplasmic pattern which enables one to explore rather directly the significance of an orderly cortical structure for cell differentiation. Because of certain pigmented stripes, the disposition of this pattern is even observable in the living animal so that transformations usually can be followed from experiment to some equilibrium state in a given specimen without sacrificing it for staining. And second, the amazing amenability of stentors to grafting and other drastic experimental manipulations permits one to combine and rearrange cortical or ectoplasmic patterns and other cell parts in ways which exhaust almost all the conceivable possibilities. Included among these operations are enucleations of the cell and transplantations of nuclei from one species of *Stentor* to another. Hence, insofar as the nucleus may be a source of critical substances, isolated by nature in the prevailing nucleo-cytoplasmic duality, the experiments here reviewed may be considered as exploring the interacting roles of pattern and substance in the differentiation of a single cell. Most of these experiments are new and will be substantiated in extended publications soon to appear.

I. MATERIALS AND METHODS

A brief outline of the morphology of *Stentor coeruleus* and note on the methods of operation employed will illustrate favorable features of this material mentioned above. Fig. 1A shows the principal parts visible in the living animal. At the anterior end is the head or feeding apparatus, and at the opposite end is the tail or holdfast which serves for temporary attachment. Running between head and tail on the surface is a series of about 100 pigmented stripes between which can be seen the rows of body cilia. There is evidence that these linear components of the cortical pattern have an intrinsic polarity which is not easily al-

[1] Associate Research Zoologist of the Department of Zoology, University of Washington. This project was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

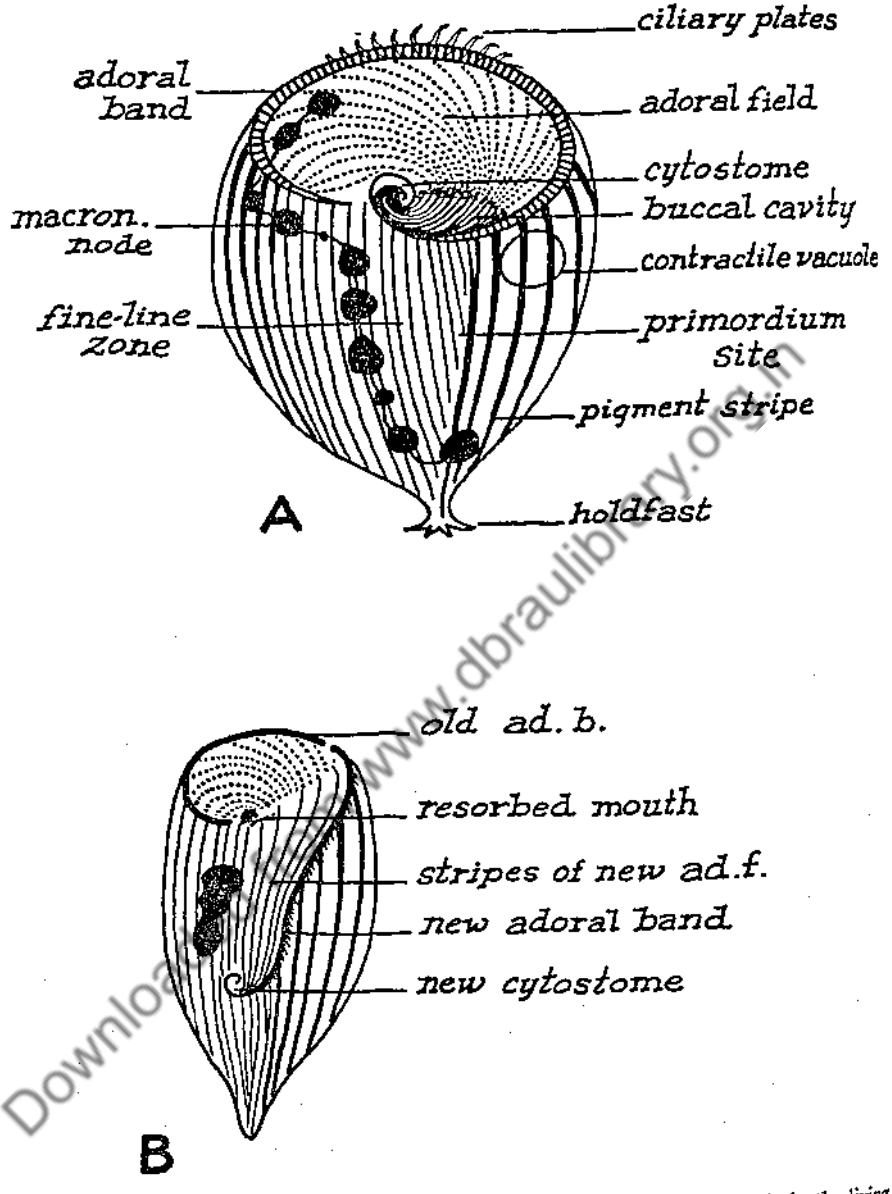


Fig. 1. A. Diagram of *Stentor coeruleus* showing principal parts visible in the living animal. B. The same, in mid-phase of reorganization. A new set of oral structures is formed at the primordium site and replaces the old, which are partly dedifferentiated. Meanwhile the macronuclear nodes temporarily fuse together.

tered (Weisz, 1951; and *vide infra*). In addition, the cortical pattern of the cell shows in its pigment stripes a left-right difference. This consists in a gradual variation in the width of these lines. Beginning some-

what to the left of the oral meridian the widest pigment stripes are found and wide striping continues around the back of the cell. As one approaches the oral meridian again, narrower stripes form what we shall call the fine-line zone, and finally narrowest stripes meet widest at a juncture which we shall refer to as the locus of contrast in stripe widths.

This juncture or place of maximum anisotropy in the stripe pattern is important for two reasons. First, it is here that the primordium of a new head always appears in division, regeneration, and reorganization (Fig. 1B). Initially the oral primordium is evident as a slightly curved white line toward the anterior end of the pole-to-pole locus of stripe contrast, as if the pigmented striping parted to permit emergence of the new organelles. Closely-packed oral cilia soon appear in this opening and begin a slow, rhythmic beating. The posterior end of the primordium then coils sharply to form the mouth, finally, the anlage curves toward the anterior pole of the original cell or of the posterior daughter cell in division, cutting off and sweeping fine stripes with it into the adoral field. This loss of lateral stripes could account for there being a normal provision for increase in number of stripes as now described.

The second point of interest about the locus of stripe contrast is that this appears to be the region where new body stripes are formed, as indeed is suggested by Schuberg's (1890) originally describing it as a "ramifying zone." Here it is frequently observed that the widest pigment stripes split from anterior to posterior, each stripe so divided resulting in two or more fine stripes; and it may be assumed that the rows of body cilia lying between these stripes also multiply in some manner. This increase in the stripe pattern is especially noticeable, as might be expected, in longitudinal-half fragments which set about promptly to recover the definitive complement of about 100 stripes, starting with half that number (see Fig. 9c). Stripes, like the whole cell itself, are apparently "born" in fission and lose their identity by division into daughter stripes. It is also to be inferred that the body stripes increase in length as the cell grows in size.

Underlying the cortex of the cell is the moniliform macronucleus which is visible in the living animal. Nuclear nodes, of which there are about 15, are located in a fairly constant position as shown in Fig. 1 and may be excised in part or totally by the use of a glass needle.

Structures not readily visible in the living animal include the micronuclei, contractile myonemes, and sub-pellicular structures associated with the origin and co-ordination of the cilia. In regard to the latter,

which have been called the infraciliature, one can see between each pair of pigment stripes a row of body cilia which undoubtedly (as in the silver-staining studies of *S. felici* n. sp. of Villineuve-Brachon, 1940) arise from kinetosomes and are joined by connecting fibers which coordinate the cilia in metachronal rhythm (Worley, 1934). Hence the colored striping may be taken as an adequate indication of the disposition also of the pattern of the infraciliature which is involved directly in the construction of oral primordia.

Underlying the ciliary fibers are the myonemes which account for the high contractility of stentor. When attached, the active head can draw out stentor to perhaps 10 times its diameter when contracted and contraction occurs with amazing alacrity. This cell is therefore also a good "muscle," hence it may be of some significance that muscle and ciliary functions occur in the ectoplasm close to morphogenetic events.

"Differentiation" in stentor may be defined specifically as the formation and development of an oral primordium; and this process is reversible since organelles once formed can later be dedifferentiated and resorbed, as occurs for example in the replacement of the mouth regions in reorganizations of the cell (Fig. 1B). The dramatic act of elaboration of the head structures is supplemented by less obvious multiplication and adjustments of the stripe pattern in the ectoplasm as well as differentiation of the holdfast.

Observations on other species of *Stentor* show that their mode of differentiation is the same as in *coeruleus* and is also related to a comparable ectoplasmic pattern.

All the operations to be described were performed by hand under a dissecting microscope using glass needles. Their possibility depends on the fusability of naked endoplasms in stentor (Tartar, 1941). Thus if a sector is cut from one cell and an incision made into another to expose the endoplasm, quick apposition of the endoplasm on the inside of the sector with that of the host will result in permanent adherence of the graft. Similarly, cells can be grafted to other cells after their endoplasms have been exposed. The animals are quieted mechanically by placing them temporarily in a viscous solution of methyl cellulose, and all operations must be done rapidly enough so that a firm membrane will not have been formed over the exposed endoplasms.

II. ORAL DIFFERENTIATION AND THE ECTOPLASMIC PATTERN

In dealing with the cortical or ectoplasmic pattern of stentor our attention naturally falls first on the sector where wide meet narrow pig-

ment stripes, since this is the site where the primordium of the head normally appears. This region can be excised and implanted anywhere within the pattern of the same or of another cell. Thus when a primordium site from one cell is grafted into the back of another, this cell has two sites and when it regenerates, two primordia and a doublet head are produced (Fig. 2) in which two sets of oral structures are oriented neatly around the anterior pole.

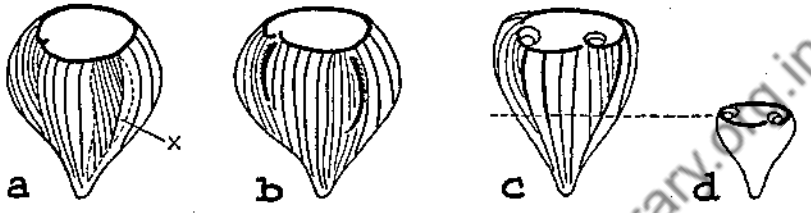


Fig. 2. Implantation of an extra primordium site leading to double oral regeneration. a. The graft (x) is inserted among the wide stripes of the host to the left of its own primordium site. b. Two primordia formed, one at each site. c. Resulting doublet stentor with two sets of feeding organelles and two primordium sites. d. Double regeneration of a posterior fragment of a doublet.

Such doublet stentors retain for weeks the two primordium sites and they divide, reorganize and regenerate as doublets. For example, a small posterior fragment regenerates as a diminutive doublet since it bears the posterior portions of two primordium sites (Fig. 2d). Doublets, however, can be reconverted into singles by the reciprocal experiment in which one of the primordium sites is excised, even though the bistomial head is left intact.

It would therefore appear that the primordium site carries a precursor of the head primordium, yet further experiments indicate at once that this is not the case. For if the site is excised from a stentor this does not prevent regeneration of the head, which may even occur as rapidly as when the normal site has not been removed. Similarly, if both primordium sites of a doublet are removed, the animal still regenerates doubly. In either case, the primordium now appears at the suture or line of heal where pigment stripes of different widths come together.

Similarly, if a sector of fine lines, just to the right but not including any of the normal primordium site, is implanted into the back of a stentor among the wide pigment stripes (Fig. 3) it reacts like a normal site. On regeneration, primordia appear both at the normal site and on the left side of the implant where its finest stripes meet the wide stripes of the host. Again, the normal primordium site is shown to be dis-

pensable in oral differentiation, which arises not from a localized precursor but appears to be due to a relational property within the cortical pattern, namely, *a condition of which the visible aspect is the juxtaposition of wide and narrow pigment stripes.*

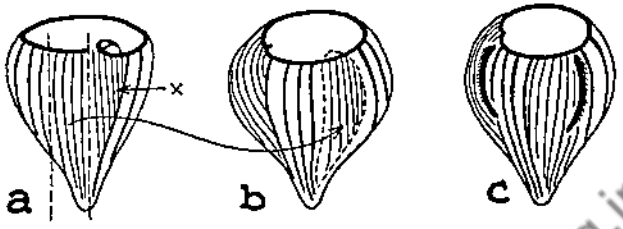


Fig. 3. Implantation of an extra fine-line zone leading to double regeneration. a. Origin of the implant from one cell, excluding the normal primordium site (*x*). b. The fine-line sector grafted between wide stripes of the host. c. Regeneration primordia at both the host primordium site and the locus where the finest lines of the implant lie adjacent to wide stripes of the host.

This empirical correlation between primordium formations and the state of the visible stripe pattern can be tested by producing all manner of appositions of wide- and narrow-stripe regions differing from that which obtains only at the primordium site normally. If the fine-line zone of a stentor is divided by the implantation of a sector of wide striping from the back of another cell, three junctures of wide and narrow striping are evident (Fig. 4) : at the normal primordium site and on

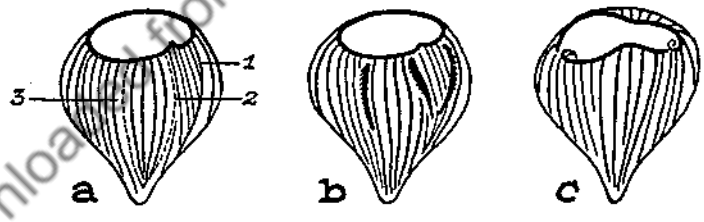


Fig. 4. Multiple primordium formation produced by splitting the fine-line zone with a sector of wide striping. a. The implant (dotted outline) results in two extra loci of stripe-width contrast (2 and 3) besides the normal host primordium site (1). b. Resulting 3-fold primordium formation. c. Doublet stentor produced, with incomplete mouth-formation in the system arising from the joining of primordia at loci 1 and 2.

each side of the implant. When the mouth is excised to induce regeneration, it is generally the case that three oral primordia appear, one at each of the loci of stripe contrast. This experiment shows not only that primordia may form at regions of apposition of wide and narrow pigment stripes even when this leads to supernumerary formations, but also that it makes little difference in the primary differentiation whether

the fine-line area lies to the right or to the left of the wide striping. In the further development of the primordia, however, tristomial heads are not produced because of the tendency of anlagen to join with each other to form fewer than three developing systems.

Control experiments show that it is the apposition of wide and narrow stripe areas which is crucial for oral differentiation. If fine-stripe sectors are implanted in fine-line zones, if wide-stripe sectors are placed in wide-stripe regions, or if the fine-line zone is split by additional narrow striping, no supernumerary primordia are formed.

The relationship between pattern and primordium formation can be explored further by making a circumferential cut around the middle of the cell and rotating the anterior half 180° on the posterior. The primordium site is then divided transversely and its two parts lie on opposite sides of the cell (Fig. 5). On regeneration, oral differentia-

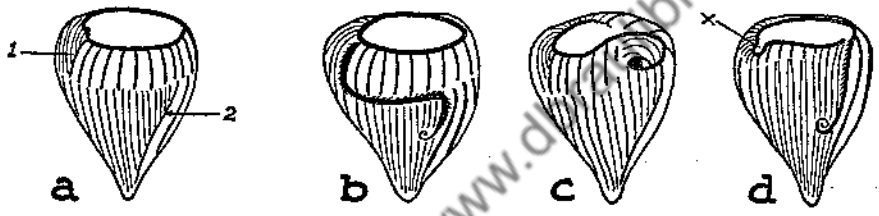


Fig. 5. Effect of rotating the anterior half of the cell 180° on the posterior half. a. The operation divides the original primordium site into two displaced sections. b. Primordium formation occurring at both parts of the primordium site and in the suture between where wide abut narrow stripes end to end. c. Resulting regenerate with mouth supplied only by the posterior half and showing displacement of anterior striping by extension of that of the posterior half. d. Subsequent reorganization with a complete primordium forming only between posterior stripes. The anterior stripes are now nearly resorbed and contribute only a notch (x) to the new oral field. Note that a pair of fine pigment stripes tend to meet but not to join with each wide stripe of the anterior component.

tion then occurs in both halves simultaneously; but it is especially interesting that the two primordia usually become connected by a transverse primordium which may extend halfway around the cell. This surplus differentiation outside the parts of the normal primordium site occurs at the suture between the cell halves *where widest stripes abut narrowest pigment stripes end-to-end.* Thus the formative relation between wide- and narrow-stripe areas is effective even when they do not lie side by side as in the normal situation.

Such cases show an additional phenomenon of interest from the standpoint of what patterns do in stentor. The disharmony in the stripe pattern is resolved by resorption of the striping of the anterior

half while that of the posterior component extends and takes over. The more anterior structures seem therefore to be the opposite of "dominant," and this is in apparent contradiction of Child's postulate of an anterior-posterior gradient of metabolic activity in stentor (for references and further criticism, see Weisz, 1948b). This eventuality is forecast by the fact that when a continuous primordium is formed a mouth appears only at its posterior terminus. In the subsequent reorganization an adequate oral primordium forms only among the originally posterior stripes and the anterior primordium is either missing or abortive, according to the state of resorption of the anterior striping (Fig. 5d).

Without describing in detail all the permutations of these experiments which have been tested, it may suffice to state that *oral primordia appear wherever and however wide- and narrow-stripe areas of the ectoplasmic pattern come together to create a locus of sharp contrast in pigment stripe widths.* The contrasting areas may abut end-to-end with opposite polarities, lie side by side in heteropolar orientation, or rest at right angles to each other—in all cases the number and location of the primordia is determined by the number and location of the loci of stripe contrast, but is independent of the manner in which wide- and narrow-stripe regions come to lie together.

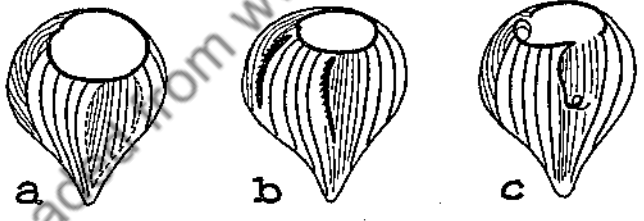


Fig. 6. Heteropolar implantation of an extra sector of fine striping. a. The operation in which the implant was rotated 180° before grafting. b. Double primordia, one at the host primordium site and one where the narrowest stripes of the implant lie next to wide stripes of the host. c. Resulting product in which the primordium in the heteropolar fine-line zone was incompletely developed and of reversed asymmetry.

The symmetry of the oral differentiations, however, often reflects the polarities and disarrangements of the manipulated pattern, as shown in the following experiment. A patch bearing the fine-line zone exclusive of the normal primordium site can be cut from one cell and implanted with reverse polarity within the wide-stripe area of another (Fig. 6). When regeneration is provoked by also excising the mouth, primordia appear both at the normal primordium site and where the finest stripes of the implant meet wide stripes of the host, which is now

on the *right* side of the patch (cf. Fig. 3). At first the ectopic anlage begins a mouth-curl toward the posterior end of the graft, but soon the polarity of the host predominates and effects an oral differentiation at the opposite end. The ingestive structure is both of reversed asymmetry and incompletely developed because the wide stripes are now on the "wrong" side of the narrow. Nevertheless, it is demonstrated that wide- and narrow-stripe areas interact to determine primordium formation even when heteropolar.

From this experiment one would expect that if the normal stripe pattern could be reversed, with the fine stripes lying to the left instead of to the right of the wide stripes, heads of reversed asymmetry would always be produced. It has happened in three instances that grafted stentors gave rise to products of this type. The case shown in Fig. 7

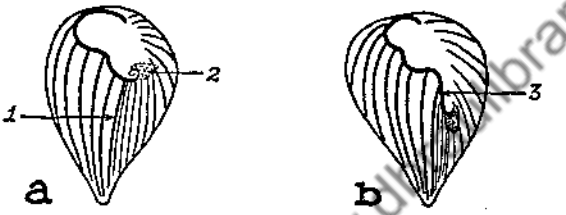


Fig. 7. A case of reversed asymmetry in stripe pattern and consequently also in the feeding organelles. a. Wide stripes lie to the right instead of to the left of the narrow stripes (1). Mouth spiral is reversed and gullet is not coiled (2). b. Subsequent reorganization primordium (3) was also of reversed asymmetry and mouth-formation still less complete.

was half of a fusion mass of ten aboral, lateral-half fragments grafted at random. Somehow, in the realignment of the graft components, this product was separated off and had a stripe pattern the reverse of the normal. It formed a mouth region of reversed asymmetry which was not perfect and the gullet was an uncoiled pouch; on reorganizing a still less perfect mouth was differentiated, also coiling in the "wrong" direction. The body-stripe pattern is therefore not only correlated with the locus of appearance of the oral primordium but also with its symmetry, though there seems to be some resistance to forming mouths of counter-clockwise spiral.

So far, we have been dealing only with oral regeneration, but the tail or holdfast is evidently also closely correlated in its formation with the visible body striping, though its elaboration is less dramatic. Wherever posterior ends of stripes come together a holdfast is there formed. More striking is the fact that a new holdfast and posterior pole may be produced under unusual circumstances which reveal unexpected po-

tentialities of the cortical pattern as shown in the following experiment. If stentors are cut in two longitudinally, the aboral halves so produced usually fold over so that head and tail remnants come together and the body striping bends sharply at the fold (Fig. 8). Along this bend the stripes later become severed, much as in the formation of a division furrow, and the newly formed termini come together in stellate arrangement to produce a new tail. In the meantime the original half-tail emerges as a subsidiary center of stripe organization, and a new set of oral structures is regenerated from the locus of greatest stripe contrast which now appears to be an apposition of the widest stripes and their own more attenuated posterior ends. Quite possibly the polarity of part of the original body striping is reversed to accommodate to the newly created posterior pole.

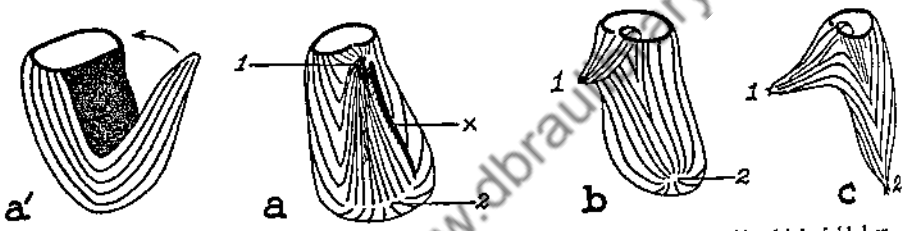


Fig. 8. Oral and caudal regeneration in a longitudinal adoral half which folded on itself in closing the wound surface. a'. Folding of half-cell fragment to close exposed surface of endoplasm. a. A primordium forms at a locus of stripe contrast where wide stripes lie next to their more attenuated posterior ends (x). Original posterior pole (1) now approximates the oral region; striping breaks at the fold (2). b. Severed ends of body striping come together to form a new posterior pole (2), while the original tail (1) organizes striping around itself. c. Resulting specimen with two posterior poles and two holdfasts and a single head.

The correlation of primordium formation with apposition of distinguishable pattern areas at once brings forth the question of how great the difference in those areas must be, as reflected in pigment stripe widths, in order that oral differentiation may occur. The answer seems to be that any appreciable difference suffices, but that as we shall soon see, the presence of other loci of stripe contrast may exert an inhibitory influence. Thus if aboral longitudinal halves be cut, so that both widest and narrowest stripes are excluded, and if such fragments do not chance to fold on themselves, there still will be a difference in the graded widths of the stripes remaining which is maximal at the line of heal. It is just here that the regeneration primordium forms (Fig. 9b). As mentioned earlier, after regeneration there is a splitting and multiplication of stripes (Fig. 9c) which eventually restores the normal number and gradation.

Theoretically, one might expect to be able to produce similar but narrower fragments in which there was not sufficient contrast of stripe widths, or whatever is correlated therewith, to determine a primordium, and in fact three instances were found in which such fragments failed to regenerate at all (Fig. 9d) though they were quite large, nucleate, and survived for many days in healthy condition.

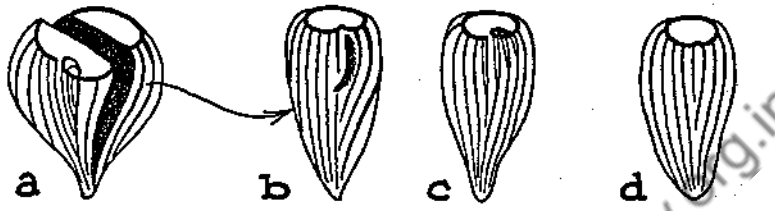


Fig. 9. Morphogenetic performance of aboral halves. a. The longitudinal half is cut to exclude original primordium site and widest and narrowest stripes. b. A difference in stripe widths nevertheless appears at the line of heal because remaining stripes are of graded widths. The regeneration primordium appears at the locus of stripe contrast. c. Following regeneration, wide stripes split into narrow stripes, thus regenerating both the normal primordium site and the normal number of stripes. d. A case in which a fragment of this type failed entirely to regenerate, presumably because of insufficient contrast in striping.

In certain types of graft complexes where there is more than one locus of stripe contrast, primordium formation at some may be suppressed. Two aboral halves fused in homopolar parabiosis lack both widest and narrowest stripes as well as a normal primordium site, yet the lines of heal of the body stripes on opposite sides show stripe-width contrast of minor grade (Fig. 10). These combinations always re-

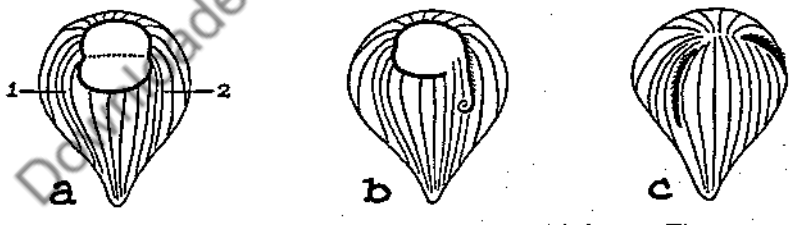


Fig. 10. Regeneration in parabiotic grafts of two adoral halves. a. The sutures form loci where wide meet less-wide stripes (1 and 2) and the two sectors of adoral band join to form one oral field. b. A primordium forms at only one of the loci and a single stentor is produced. c. Alternatively, if the adoral bands are removed primordium formation occurs at both sutures and a doublet stentor is produced.

generate singly, a primordium appearing only on one side. That both loci of stripe contrast can give rise to primordia is, however, proved by the experiment in which the remnants of the adoral bands are re-

moved since then, for some reason not yet known, double regeneration occurs in the majority of cases (Fig. 10c). When one normal primordium site of maximum stripe-width contrast is grafted to two such aboral halves this site takes precedence over loci of less contrast and only one primordium is produced. The same is the case with complexes of three and even more aboral halves plus one primordium site; in their initial regeneration a primordium usually appears only at the normal site and giant singles are formed. But in these cases, in which the size of the complex is greater than normal, subsequent reorganization may show activation at some of the sutures which had previously been quiescent, and doublet or triplet heads then arise. Fig. 11 below

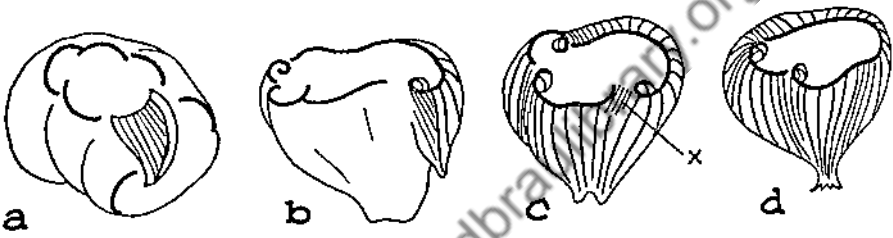


Fig. 11. Dominance and subordination in multiple loci of stripe-width contrast. a. Graft of 6 aboral halves and one normal primordium site of maximum stripe contrast. b. Complete oral regeneration initially only from the normal site; indications of partial regeneration at two sutures between the aboral halves. c. Reorganization as a triplet with 3 loci activated; resorption of fine lines (x) of original primordium site evident. c. Re-reorganization as a doublet with primordia from two sutures but none where original primordium site disappeared.

illustrates an instance of this type. One normal primordium site was implanted into a fusion mass of six aboral halves. Complete oral differentiation occurred initially only at the implant, but in subsequent reorganization a triplet head was produced. Finally, the fine lines of the original primordium site were apparently resorbed and the next reorganization yielded a doublet with primordia arising only at two of the sutures between the grafted halves.

Hence there seems to become established in any graft complex an intimate relatedness of the pattern components such that suppression of oral differentiation at loci of less sharp stripe-width contrast may occur in certain arrangements because of the presence of regions of greater contrast, though subsequent release and even exchange of dominance may later take place.

Even when two or three equivalent primordium sites are present a regulation toward the normal single type eventually takes place. This adjustment is most clearly evident in graft products with doublet or

triplet heads. These forms may divide, reorganize, and regenerate as doublets and triplets for a considerable period of time and might therefore be called biotypes; but eventually the triplets become doublets and all doublets finally produce singles. Although it is only by rare chance that one happens to catch these forms in the act of reducing their oral valency, the reduction is apparently brought about by gradual resorption or obliteration of one primordium site (as indicated Fig. 11c), whereby incomplete oral differentiation may then occur at this site and later none at all. It is certainly the case that singles produced from doublets and triplets have only one locus of stripe anisotropy. The number of oral differentiations is therefore a function both of the number of loci of stripe contrast and regulative forces within the pattern which tend toward recovery of the normal, single type.

Multi-individual complexes in other ciliates, resulting from natural or induced abortion of cell division, have long been known and have shown the relative persistence of these complexes as well as their capacity often to return to unitary individuality (see Fauré-Fremiet, 1945 and 1948; Sonneborn, 1932; and Kimball, 1941).

III. REGENERATION OF FRAGMENTS

The relation of oral differentiation to what is empirically observable as lines of apposition of wide and narrow pigment stripes will now explain in terms of cytoplasmic pattern why it is usually impossible to cut a nucleate fragment of stentor which will not regenerate. Since the parent

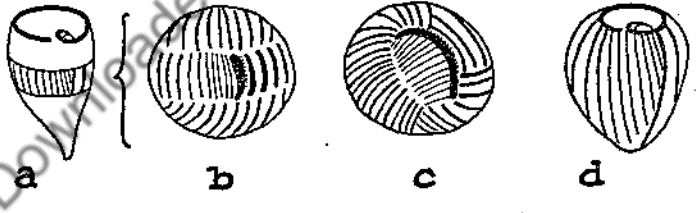


Fig. 12. Regeneration in a small stentor fragment. a. Origin of fragment: a disc from the mid-region of the cell (illustrated in reduced scale). b. Folding of striping to cover wound surface and appearance of regeneration primordium at the remaining, short section of the original primordium site. c. Enlargement of the primordium and beginning reorientation of striping. d. Regeneration of a normal stentor, one day after the operation.

pattern consists of polarized stripes of graded widths with a locus of stripe-width contrast, any such fragment (see Fig. 9b) will also bear a cortical pattern which can be described in the same words: a patch of polarized stripes of graded widths which, on healing, brings wide and less

wide stripes together in a locus of stripe contrast sufficient to determine a regeneration primordium. The regenerated head will also be generally proportionate to the size of the fragment since the locus of stripe contrast will also be shorter in smaller fragments, and there is thus a quantitative relationship between the length of the locus and that of the oral primordium. For example, in middle slices of stentor (Fig. 12) the small remaining collar of striping doubles over to cover the exposed wound surfaces and the primordium appears in the small section of the primordium site contained in such a fragment. This explanation therefore removes much of the magic if not the wonder associated with the regenerative performance of stentor; for it is clear that although a fragment is initially only part of the original individuality, it carries with it the essential features of the pattern of the whole.

IV. MINCED STENTORS

A stentor can be cut into a multitude of viable pieces if the fragments are not separated but allowed to adhere by their endoplasm and to participate mutually in the support afforded by the nucleus, but then they no longer behave independently (Tartar, 1941). With a glass needle the entire ectoplasmic pattern is cut into tiny patches too small for further sectioning which lie in random orientation, looking much like furrowed fields seen from the air (Fig. 13a). Yet even such drastic interference does not defeat the pattern factor, which is not dedifferentiated but sets about to mend itself.

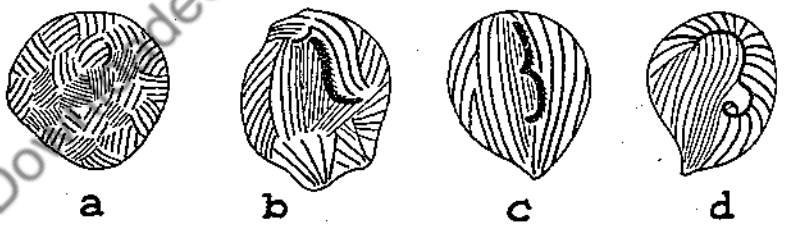


Fig. 13. Morphogenesis in a minced stentor. a. All of the original oral structures have been removed except one short piece of the adoral band; pattern cut into many disarranged patches. b. Beginning alignment and rejoining of patches with a somewhat irregular primordium appearing at an abnormal locus of stripe-width contrast. c. Further development of the primordium and realignment of stripes. d. Nearly completed regeneration of a normal, single stentor with only slight disharmonies in stripes remaining.

In these cases the patches slowly reorient themselves with respect to one another so that areas of parallel striping appear and the stripes then join together. These seem to be the two tendencies at work—reorienta-

tion and joining—for the patches do not all realign at once like magnets. After a few hours one, or even two, loci of stripe-width contrast appear and oral regeneration begins, though the primordium may follow a rather devious course owing to the still patchy character of the pattern (Fig. 13b). Oral individuation may thus be realized even within the normal regeneration time of 5 hours, but considerable disorientation of stripes usually persists in parts of the cell for some time.

An orderly pattern of pigment stripes is therefore reconstituted by the parts of the original pattern realigning themselves with respect to each other and joining together in stripe continuities without losing their original character as parts. There is no extensive migration of patches but only torsion in situ. Oral regeneration occurs when a sufficient locus of stripe contrast has been established, long before all patches are properly organized. How many such loci are formed seems to be a matter of chance. Usually a single minced stentor will form one only but it can produce two if the patches are sufficiently displaced in the cutting. Similarly, minced doublets with two original loci of stripe contrast generally regenerate doubly, apparently for the reason that in them there is a greater chance for the fine-line patches to join together in two groupings. Polystomial stentors hence do not seize the opportunity of the chaotic and labile state produced by mincing to integrate into the single, normal type. Of interest, too, is the fact that if a complete head is grafted onto a minced stentor, no regeneration occurs, so that even a patchy pattern can "communicate" that oral regeneration is not called for.

V. STENTOR MASSES

An indefinite number of stentors can be grafted together into one protoplasmic continuum. As many as 100 individuals have been fused into a single mass (Tartar, 1954). The viability of these fusion complexes is not greatly reduced in spite of their large size; for they live about a week, the same as starved normal animals, which is ample time for the manifestation of considerable morphogenetic behavior. Though often much larger than normal, masses do not resort to multiple fission to split up at once, so that the initial situation created by grafting generally maintains and can be studied.

Stentor pairs have been grafted together in a variety of arrangements, but there is no orientation which precludes their attaining the single, unitary shape. Even cephalobiotics can close jack-knife fashion to form singles, and in pairs grafted by the tails one member may become largely incorporated into the other. The cortical patterns of the two components

therefore tend to coordinate to produce a unified shape, and shape at any time is strictly an expression of the arrangement of the body striping.

Grafted pairs or 2-masses may in oral regeneration produce the single-headed type at once, but in the majority of cases they regenerate doubly or produce the doublet type (Fig. 14a). In a few instances hyper-regeneration occurred, or the formation of three sets of feeding organelles. This range of morphogenetic behavior may now be interpreted in accordance with the correlation of oral differentiation with loci of stripe-width contrast. For in the majority of cases it could be expected that although the stripe patterns join and become parallel, the two original primordium sites remain unchanged so that regeneration would be double; in fewer instances one primordium site may have been cut into and used as the line of fusion so that it became degraded in stripe contrast in reference to an undisturbed primordium site where oral regeneration alone occurred; and finally, though more rarely, as in the experiments already described in which the fine-line zone was split by a section of wide striping, new loci of stripe contrast in addition to the original two primordium sites were probably created which led to the triple regeneration.

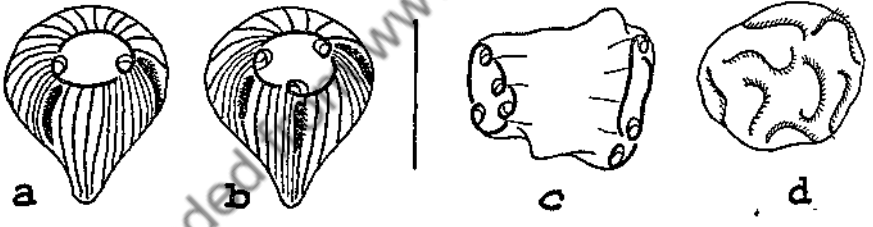


Fig. 14. Forms produced by fusion masses of stentors. a. Doublet stentor from a 2-mass, reorganizing as a doublet. b. Triplet from a 3-mass, reorganizing as such. *In reduced scale:* c. A mass of 14 stentors did not recover normal shape and formed a reduced number (7) of oral structures. d. A mass of 55 stentors with no shape regeneration, only 8 oral differentiations and these merely forming garlands of aboral cilia without mouths.

The results with 3-masses were similar. Again there was a correlation of stripe patterns toward the attainment of unitary shape, but this integration was now more difficult and required a longer period of time. Many 3-masses regenerated at once as doublets, the majority as triplets (Fig. 14b), and a few as quadruplets. The doublets and triplets were self-reproducing and could maintain themselves for weeks as polystomial biotypes, but it is interesting that quadruplets were temporary formations which tended to reduce promptly to triplets.

The latter point is emphasized by the morphogenetic performance of 4-masses. Although these complexes could express four-fold regeneration at first, they then promptly reduced the oral valency. It was also the case that some regenerated singly at once with only one head primordium appearing, though this never occurred in the 3-masses. As the number of components in the mass is increased there is a tendency toward reduction of heads, and we may infer that the formation of primordia at some of the original primordium sites was suppressed. Hence grafts of four stentors may show at once what is only brought about gradually in the doublet and triplet biotypes, namely, that fusion masses tend to reduce the number of sets of oral structures to unity by the obliteration of all but one primordium site or locus of stripe-width contrast. In the course of this reduction a primordium site may form only a short primordium or a primordium which is incomplete in that it lacks the mouth, then later none at all. Masses of more than four stentors never regenerated as many heads as the number of components but invariably showed a reduction in oral valency (Fig. 14c).

Grafts of four also inaugurate another tendency in fusion complexes: a loss of the capacity for complete and normal oral differentiation. In these and even more so in larger aggregates, regeneration often leads only to the formation of long garlands of oral cilia which lack a cytostome entirely and no longer coil to form typical adoral bands enclosing an adoral field (Fig. 14d).

Firmly coalesced fusions of 15 or more stentors not only fail to complete the development of their oral primordia but also fail to attain anything like the normal trumpet shape. Body striping of the constituent patterns does not align parallel throughout and holdfasts are not maintained. Evidently stentor is not capable of organizing a mass and integrating a set of ectoplasmic patterns far exceeding in size the normal limits. That this breakdown is merely physiological seems unlikely since there is no progressive decrease in viability of masses with increasing size and they live as long as or even longer than unfed controls. Rather, it would appear that strains are placed on the ectoplasmic pattern as such which it is finally unable to cope with—possibly involving a mutual cancellation of polarities among the constituent patterns. In large stentor masses, therefore, we see the reverse of the increase in organization with increasing size of induced explants in amphibia and of dissociated sponge cells, a contrast which suggests that multicellularity was the only way of exceeding size limits in the pattern of single cells.

VI. NUCLEO-CYTOPLASMIC INTERACTION

The formation of regeneration primordia is correlated not only with the disposition of the cortical pattern but also with some essential contribution of the macronucleus. Early experiments with stentor (see Balamuth, 1940, Table I, pp. 302-3) were in fact among the first to demonstrate the indispensability of the nucleus for both the morphological regeneration and the continued life of the cell. Even when an oral primordium has already been formed, removal of the nucleus arrests its further development. One may suppose that the nucleus supplies some substance(s) necessary to differentiation of the cytoplasm and that this contribution is not stored, as it seems to be in *Acetabularia* (Hämmerling, 1943), so that the continued presence of the nucleus is required. In support of this assumption Weisz (1949) has observed vesicles in the macronucleus of stentor which were apparently breaking through the nuclear membrane to liberate their contents into the cytoplasm.

The presence of the nucleus is, then, essential to oral differentiation. By nucleus we mean the macronucleus, since Schwartz (1935) has demonstrated that removal of the micronuclei is without effect on the vegetative life of stentor. This circumstance is fortunate because the tiny micronuclei are obscure but the chain of macronuclear nodes, like the outline of the cortical pattern, can easily be seen in the living cell. There is no indication that the macronucleus is regenerated from stray micronuclei.

By grafting together only those parts of several stentors in which the nucleus is located, masses can be produced in which the nucleo-plasmic ratio is many times the normal value. This hypernucleate condition seems to be without significant effect: the time required for oral regeneration is not shortened but, if anything, extended, nor is its course in any way abnormal as observed. Hence the general rule for maintenance of type in polyploid cells is followed. But giant cells are not produced; instead, the excessive number of nuclear nodes is gradually reduced to normal, whether by resorption or distribution to daughter cells we do not yet know.

The relative proportion of macronucleus can be varied in the other direction by removing all but one or two nuclear nodes from the cell. Even greater reduction of the nucleo-plasmic ratio is obtained by then grafting enucleate stentors to this cell. One intact macronuclear bead is sufficient for regeneration of the head. Dwarfs are not produced and the nucleus grows and nodulates to form a normal chain. But since this increase occurs only after a few days, regeneration with reduced nuclear complement can be studied.

It was found that oral regeneration is retarded when only one or two nuclear nodes are present; both the time for the initial appearance of the primordium and period required for its complete development are extended, thus implying not only that the nucleus is required for construction and elaboration of the primordium but also that a reduced source of the nuclear contribution to this process could decrease the rate. Hence there does seem to be some significance in the nucleo-plasmic ratio in ciliates but this effect appears clearly only in the highly exaggerated reduction of the ratio which can be produced in stentors.

VII. PERFORMANCE OF ENUCLEATE STENTORS

When all macronuclear nodes are teased out of a *Stentor coeruleus* the cell lives and remains alive for the same period of time as unfed normal control animals, i.e. about 5 days. Hence enucleates are manifestly capable of whatever metabolism is associated with ciliary activity, myoneme contraction, and short-term cell maintenance. The immediate cause of death therefore seems to be merely starvation or the exhaustion of food reserves. That there are such reserves was shown by Weisz (1948a): well-fed stentors show distinguishable accumulations at the posterior pole of the cell which cytochemical tests indicated to be paraglycogen. These reserves are clearly visible in dark-field illumination as a milk-white mass. In starving stentors this mass gradually disappears and death follows a day or so later. Enucleates also utilize these reserves, but the rate of disappearance is about half that in nucleate cells. Hence it is even the case that enucleates may outlive starving nucleate controls.

The failure of enucleates to form an oral primordium and regenerate the head is hence not due to the absence of all energy metabolism nor to inability to use the carbohydrate reserves which Weisz (1948a) reports to be necessary for regeneration. It would therefore appear that the nuclear contribution is essential to specific constructive processes. Conversely, enucleates can dedifferentiate oral structures already present but this occurs late and only shortly before death. Nucleates, however, maintain their feeding organelles into death when starved, so it is indicated that the nucleus also plays a part in maintaining these differentiations.

We are particularly interested in any morphogenetic capacities or pattern activities which may be expressed even in the absence of the nucleus. From recent experiments it can be stated that enucleate *S. coeruleus* can (1) regenerate the holdfast, (2) form a new contractile vacuole, (3) heal the ectoplasm after cutting or grafting, and (4) align minced pattern patches in parallel and rejoin severed pigment stripes. Harmonization

of disordered striping is, however, not as certain as in nucleate stentors and some patchiness of the stripe pattern may long remain. Enucleates can do much with their cortical pattern short of the major structurization of the oral primordium.

VIII. INTERSPECIFIC COMBINATIONS

The capacities of the enucleate cell can now be compared with its performance when the nucleus of a different species is added. Nuclear transplantations are made by dissecting and isolating a few macronuclear nodes which are left enclosed in a thin envelope of endoplasm by which they may be fused to the exposed endoplasm of enucleated cells. Alternatively, a nucleated piece of one species with more than negligible cytoplasm, may be grafted to the enucleated cell of another; but then the effect of the "contaminating" cytoplasm should be separately determined. Controls in which the nucleus of one race of *coeruleus* was transplanted into another with complete recovery showed that these operations successfully transfer viable nuclei.

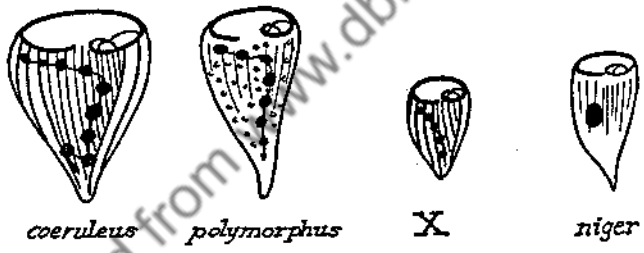


Fig. 15. Diagrams of several species of *Stentor*. *S. coeruleus*, large blue-green stentor, subject of most of this study. *S. polymorphus*, somewhat smaller stentor without pigmentation but with symbiotic algae (Chlorella). Species-*X*, possibly a new species; blue-green in color and like *coeruleus* in many other respects but much smaller. Note similar gradation in width of pigment stripes. *S. niger*, yellow-brown stentor with macronucleus a single sphere.

Some of the species of *Stentor* available for crossing are sketched and briefly described in Fig. 15. In all combinations so far tested the endoplasms of different species readily fuse and remain united. Extension of the work on stentor morphogenesis into this province has so far been largely exploratory and is complicated by such features as general necrosis of the material. Nevertheless provisional results are provocative.

Coeruleus × *species-X*. These blue-green stentors are very similar except that the unnamed species never attains a size of more than about one-sixth the volume of *coeruleus*. There is a moniliform macronucleus in both and, unfortunately, the feeding organelles have the same visible

construction though they are proportionate in size to the respective cells. The smaller species has about 48 pigment stripes compared with 100 in *coeruleus;* the stripes are about the same width, however, and species-*X* also shows a fine-line zone and a locus of contrasting stripe widths. That we are dealing with two distinct species is implied by the fact that mixed grafts did not survive.

Either nucleus when transplanted into the enucleate cytoplasm of the other species could support complete oral regeneration but these combinations did not survive. The reciprocal experiments are illustrated in Figs. 16 and 17. The size of the head is that of the species furnishing the ectoplasmic pattern; hence species-*X* nucleus can contribute to the formation of oral structures larger than those normally produced in its own cytoplasm. But since the structure of the organelles in the two species could not be distinguished, one cannot attribute a qualitative difference to either nuclear or cytoplasmic influence.

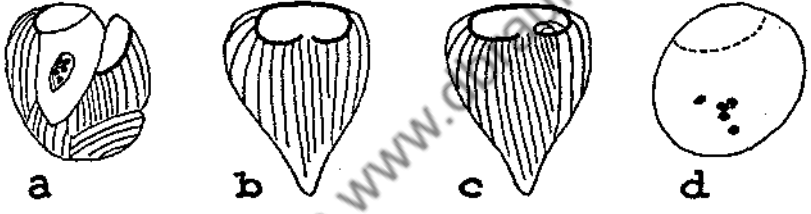


Fig. 16. Nucleus of species-*X* supporting regeneration in enucleate *coeruleus* cytoplasm. a. Small patch containing several macronuclear nodes fused to exposed endoplasm of a previously enucleated *coeruleus*. b. Reorientation of *coeruleus* striping. c. Regeneration of the feeding organelles. d. Eventual dedifferentiation and death of the combination, showing the persisting foreign nucleus.

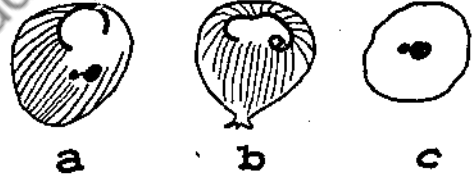


Fig. 17. Nucleus of *coeruleus* supporting regeneration in enucleate cytoplasm of species-*X*. a. Enucleated cell with two nodes of *coeruleus* implanted. b. Resulting regeneration of the oral structures. c. Subsequent death of the specimen with *coeruleus* nucleus persisting.

When whole cells of both species were grafted together a neat integration of the two stripe patterns occurred with the result that a single stentor shape was formed (Fig. 18). The feeding organelles frequently met and joined together to form a doublet head, and nuclei of both

species persisted in the complex. When the mouths of both components were removed, regeneration occurred simultaneously on both sides of the graft. However, it has been the case so far that these complexes never survived for long and did not undergo fission or further reorganization as in homoplastic 2-masses. It appears that a subtle incompatibility eventually defeated a system which otherwise functioned very well.

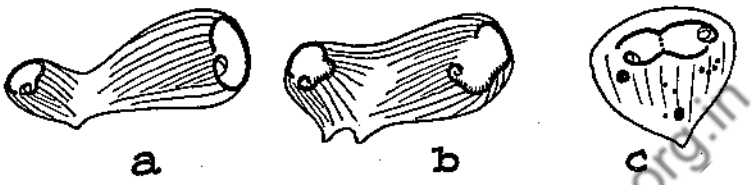


Fig. 18. Coordination of graft of one *coeruleus* and one individual of species-X. a. Animals fused at the posterior poles. b. Simultaneous reorganization on replacement of oral regions on both sides; beginning integration of shapes. c. Oral structures fusing to form doublet head, stripes parallel and integration to single, trumpet shape completed. The specimen then died.

Coeruleus × *polymorphus*. *Stentor polymorphus* has the same form as *coeruleus* but is somewhat smaller and is not self-pigmented. Body striping is therefore not easily visible and the mouth region lacks the dark pigmentation typical of *coeruleus*. Symbiotic Chlorellae usually abundant in the cytoplasm, give *polymorphus* a grass-green color and obscure the macronuclear nodes. Many of the experiments combining these two species have previously been reported (Tartar, 1953).

Polymorphus nuclei with minimum endoplasm were transplanted into enucleate *coeruleus* to test the capacity of the foreign nucleus to support oral regeneration. *Polymorphus* nucleus did not support successful oral regeneration in *coeruleus* cytoplasm; instead there resulted the prompt disappearance of remaining oral differentiations (the adoral band), as shown in Fig. 19c. The alien nucleus and cytoplasm were therefore incompatible, but the *polymorphus* nuclear beads persisted in *coeruleus* cytoplasm and had an effect there which was the reverse of maintaining oral structures. This earlier conclusion has now to be supplemented by results of more recent experiments in which different races of both species were used. Also, *coeruleus* animals were selected which had been well-fed in order to favor regeneration if possible. Great care then had to be taken to remove all the macronucleus, but controls showed that enucleation was successful even under these circumstances in at least 80% of cases. When the test was so repeated it was found that in 8 out of 9 instances transplanted nuclear nodes of *polymorphus* did enable *coeruleus*

cytoplasm to form a regeneration primordium which (Fig. 19b′) however was then obliterated. This result agrees with the former conclusion in that *polymorphus* nucleus promotes dedifferentiation or breakdown of oral structures in *coeruleus* cytoplasm, but now indicates that in combinations of certain races of these species the foreign nucleus can support an initial effort at regeneration, even to the formation of ciliary plates, which is later canceled. A situation similar to that in *coeruleus* × species-*X* is thus indicated and in stronger degree, viz., that although alien cytoplasm and nucleus interact to make possible the beginning of oral regeneration, this co-ordination in morphogenesis sooner or later gives way to an incompatibility, possibly of an immunological nature, which results in necrosis.

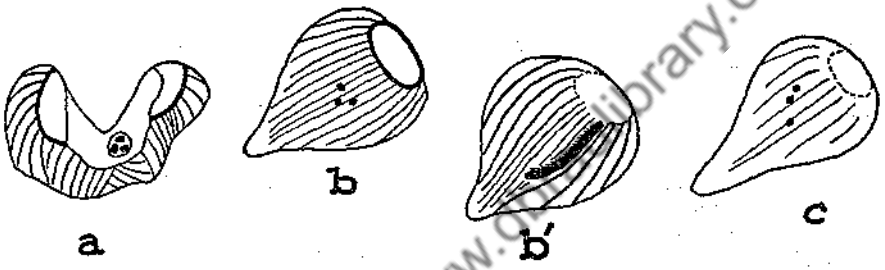


Fig. 19. Failure of *polymorphus* nucleus to support complete regeneration in *coeruleus* cytoplasm. a. Transplantation of a few *polymorphus* macronuclear nodes, surrounded by endoplasm, into an enucleated *coeruleus*. b. Recovery of orderly stripe arrangement. b′. Alternatively, a temporary primordium also formed, without further development. c. Disappearance of oral differentiations; persistence without growth of the *polymorphus* nucleus.

Many combinations were made in which *polymorphus* and *coeruleus* were grafted together in varying proportions of the different nuclei and cytoplasms. The general conclusion which emerged is that complete regeneration and survival of the chimeras occur only when there is a preponderance of the nucleus of one species in a preponderance of its own cytoplasm. For example, regeneration is not effected if grafts are prepared in which *coeruleus* cytoplasm preponderates but only *polymorphus* nucleus is present (Fig. 20). This case illustrates two further points: (1) the stripe patterns of *polymorphus* and *coeruleus* can integrate in such a graft to produce a normal stentor shape and (2) that scattering of the symbiotic algae attests a uniform mixing of the endoplasms. When one species predominates in both nucleus and cytoplasm, it regenerates a stentor of its type which can survive, and the other component is then not sloughed but somehow worked into the product. However, as the two

species components approach equality, oral regeneration is increasingly retarded or incomplete and incompatibilities finally appear which lead only to resorption of existing feeding organelles and death of the chimera (Fig. 21). It is of considerable interest, nevertheless, that the two stripe patterns reorient and so harmonize as to recover a normal, single stentor shape.

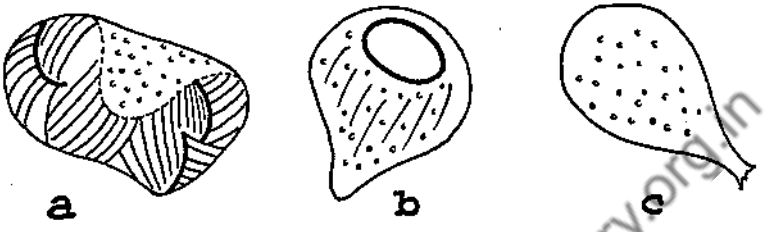


Fig. 20. Shape regeneration but failure of oral differentiation in a graft of two enucleate *coeruleus* cells and the posterior half of one nucleated *polymorphus*. a. Fusion mass following operation. b. Integration of all components into single stentor shape; scattering of Chlorellae throughout the mass. c. Dedifferentiation of pre-existing oral structures without subsequent regeneration since there is a preponderance of *coeruleus* cytoplasm and only *polymorphus* nucleus.

Fig. 21. Shape regeneration in spite of blockage of oral regeneration in graft of a whole *coeruleus* with a whole *polymorphus*. a. The operation. b. Integration of shape though components were originally at right angles to each other. c. Dedifferentiation of oral structures without regeneration. d. Death of the product.

This apparent compatibility of the cortical patterns in effecting an orderly arrangement together is further emphasized by the finding that patches of *polymorphus* grafted to *coeruleus* may result in the production of an extra set of oral structures bearing the characteristic blue-green coloration of the latter species (Fig. 22). It is possible that the implant either provides an extra primordium site in itself or causes one to be produced by disturbance of the *coeruleus* striping. We cannot decide between these alternatives because the stripe pattern was not followed, yet this much seems certain: that single *coeruleus* could be caused to regenerate doubly by addition of a minor *polymorphus* component. Such "inductions," though transitory, suggest that cortical patterns in stentor are generic and cross-active.

Coeruleus × *niger*. *Stentor niger* is characterized by an over-all yellow coloration not confined to pigment stripes. Body striping of some sort is nevertheless visible in the living cell. This species is much smaller than *coeruleus* and the macronucleus is but a single large sphere. Experiments have been limited by the fact that *niger* could not be maintained in culture.

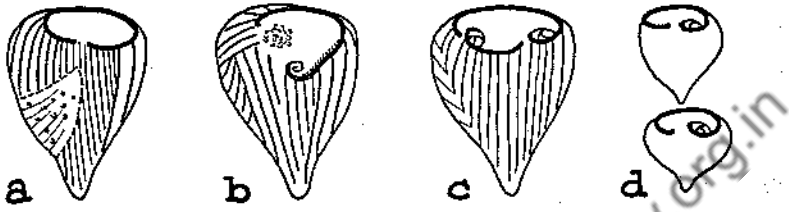


Fig. 22. Double oral differentiation produced in a *coeruleus* by implant of a patch of *polymorphus*. a. The operation; mouth removed to cause regeneration. b. Initial single regeneration at the host primordium site. c. Reorganization now with the formation of two sets of feeding organelles. d. Return to the single type on subsequent division.

When *niger* nucleus was transplanted with minimum cytoplasm into enucleate *coeruleus* no primordium formation occurred. This foreign nucleus was therefore unable to support regeneration in *coeruleus*. The *niger* nucleus, unlike that of *polymorphus*, did not cause prompt destruction of adoral bands already present; these were dedifferentiated only later, before death, as in enucleates of *coeruleus*.

Whole cells of *niger* and *coeruleus* did not behave in chimeras as do combinations with *polymorphus*. A minor, nucleate *niger* component of only one-sixth or less of the total volume was sufficient either to block regeneration or to render the development of the *coeruleus* primordium incomplete to the extent of lacking the mouth or having an oral structure which was not fully formed. Evidently *coeruleus* cannot overcome the influence of a contaminating *niger* graft. In one odd case of this type, the complicated interactions involved in this combination are suggested by the fact that a division line cutting all stripes in two was produced without subsequent cell fission.

Enucleate cells of *niger* were implanted as sectors of foreign striping in the wide stripe region of nucleate *coeruleus*. The implant delayed the regeneration of the host, but it was most interesting that when regeneration did take place there occurred in many instances the formation of *two* primordia, one at the host primordium site and one where the fine striping of the *niger* implant lay adjacent to wide stripes of the *coeruleus*

(Fig. 23). In one instance double regeneration was followed by double reorganization, but in no case was the development of the unusual primordium at the implant complete, for the mouth was lacking. The *coeruleus* nucleus therefore was apparently unable to support complete regeneration in the *niger* patch. Since the extra primordium in these cases arose at the edge of the implant, it may be inferred that wide-stripe

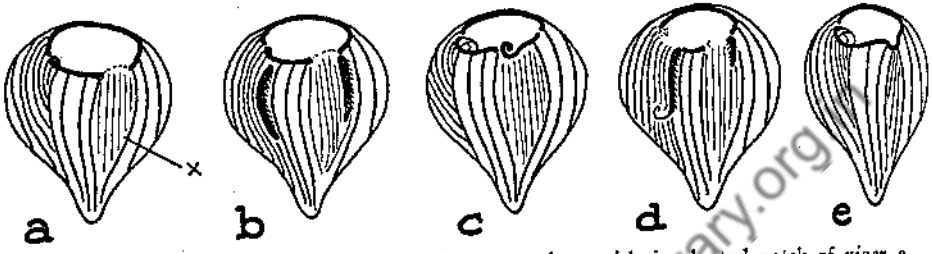


Fig. 23. Double oral regeneration in a *coeruleus* with implanted patch of *niger*. a. Enucleate *niger* grafted as a sector in the wide-stripe region of *coeruleus*, mouth of the latter excised. b. Two oral primordia, one in the host primordium site and one at the edge of the *niger* implant. c. Complete development of host primordium only. d and e. Double reorganization with still less complete oral differentiation at the *niger* implant.

areas of *coeruleus* can cause primordia to be formed in narrow-stripe areas of even a different species. Again, as in the supernumerary head-formations found with *polymorphus* implants, these results point to a generic character of the cortical pattern in *Stentor*.

IX. SUMMARY

Studies on ciliates have shown that a cell may bear a complex cortical pattern which plays a primary role in its morphogenesis. In stentor this cytoplasmic pattern factor is passed on from generation to generation in the ectoplasmic striping which is divided between the daughter cells, so that it need not be derived from some self-aggregating capacity of protein molecules. Other cells and eggs do not show such an explicit architecture, but it is not impossible to imagine the ciliate pattern being stripped of its specialized structural units and organelles, leaving a highly flexible but determinative network, capable of developing polarities and anisotropies and of guiding the course of differentiation, which might be present in all cells.

The homoplastic experiments on the cortex of *Stentor coeruleus* show that a cytoplasmic pattern, in its relational properties, can be crucial in determining the number and location of cell elaborations. To the self-reproducing components of this pattern may also be attributed the

structural elements of these differentiations, since ciliary basal bodies and pigmented stripes divide and show genetic continuity. These stripes, which we can study in the living cell, are capable of growth, multiplication, sectioning, polarization, reorientation and regulation, which together constitute considerable morphogenetic prowess. We may anticipate that many of these performances are shared by the infraciliature of stentor. Therefore it is possible that the complex activities of the cyto-architecture of stentor may forecast an appreciation that some homologous cytoplasmic pattern is common to all cells and is as important in its way as the chromosomal nucleus which also has its orderly arrangements.

The relationship of cortical pattern to cell differentiation revealed in the experiments with stentor is at present a purely empirical correlation between oral primordium formation and the juxtaposition of areas characterized by wide and by narrow pigment stripes. Precisely what occurs at these loci of contrast in stripe widths has not yet been determined. It is of course highly improbable that the pigment stripes are anything more than the visible markers of differences between different parts of the infraciliature from which the primordium is directly constructed. Therefore it remains an open question whether wide-stripe areas "induce" primordia in narrow-stripe regions adjacent to them or whether the two contrasting regions collaborate in these formations.

Heteroplastic grafting shows that the cytoplasmic patterns of different species of *Stentor* are not only visibly similar but can co-ordinate in shape regeneration and are at least partially cross-active in primordium formations. It may therefore be possible that in these cortical patterns lies the generic agent responsible not for the species characterization of the parts but for the general form of the organism, to which the nucleus may add an influence which effects species differences while also making possible any differentiation at all.

In stentor, nuclei can often support primordium formations in foreign cytoplasm. Whether these elaborations follow the species type of the nucleus or that of the cytoplasm we cannot yet say since the oral structure in different species of *Stentor* is so similar. But it has not been found that foreign nuclei support continued life of the cell. Hence it would seem that a common system of nucleo-cytoplasmic interaction in morphogenesis within the genus is canceled or obscured by subtle and pervasive conflicts between the protoplasms of different species.

BIBLIOGRAPHY

Balamuth, W. 1940. Regeneration in protozoa: a problem of morphogenesis. *Quart. Rev. Biol. 15,* 290-337.

Fauré-Fremiet, E. 1945. Symétrie et polarité chez les Ciliés bi- ou multi-composites. *Bull. Biol. Fr.-Belg. 79,* 106-150.

Fauré-Fremiet, E. 1948. Doublets homopolaires et régulation morphogénétique chez le cilié *Leucophrys patula. Arch. anat. microscop. morphol. exp. 37,* 183-203.

Hämmerling, J. 1946. Neue Untersuchungen über die physiologischen und genetischen Grundlagen der Formbildung. *Naturwiss. 12,* 337-342, 361-365.

Kimball, R. F. 1941. Double animals and amicronucleate animals in *Euplotes patalla* with particular reference to their conjugation. *J. Exp. Zool. 86,* 1-32.

Schuberg, A. 1890. Zur Kentniss der *Stentor coeruleus. Zool. Jahrb. Abt. Anat. 4,* 197-238.

Schwartz, V. 1935. Versuche über Regeneration und Kerndimorphismus bei *Stentor coeruleus* Ehrbg. *Arch. Protistenk. 85,* 100-139.

Sonneborn, T. M. 1932. Experimental production of chains and its genetic consequences in the ciliate protozoan, *Colpidium campylum* (Stokes). *Biol. Bull. 63,* 187-211.

Tartar, V. 1941. Intracellular patterns; Facts and principles concerning patterns exhibited in the morphogenesis of ciliate protozoa. *Growth 5* (supplement), 21-40.

Tartar, V. 1953. Chimeras and nuclear transplantations in ciliates, *Stentor coeruleus* × *S. polymorphus. J. Exp. Zool. 124,* 63-104.

Tartar, V. 1954. Reactions of *Stentor coeruleus* to homoplastic grafting. *J. Exp. Zool. 127,* 511-576.

Villeneuve-Brachon, Simone. 1940. Recherches sur les ciliés heterotriches. *Arch. Zool. exp. gén. 82,* 1-100.

Weisz, P. B. 1948a. The role of carbohydrate reserves in the regeneration of *Stentor* fragments. *J. Exp. Zool. 108,* 263-278.

Weisz, P. B. 1948b. Regeneration in *Stentor* and the gradient theory. *J. Exp. Zool. 109,* 439-450.

Weisz, P. B. 1949. A cytochemical and cytological study of differentiation in normal and reorganizational stages of *Stentor coeruleus. J. Morph. 84,* 335-364.

Weisz, P. B. 1951. An experimental analysis of morphogenesis in *Stentor coeruleus. J. Exp. Zool. 116,* 231-257.

Worley, L. G. 1934. Ciliary metachronism and reversal in *Paramecium, Spirostomum,* and *Stentor. J. Cell. Comp. Physiol. 5,* 53-72.

V. INFECTION AND HEREDITY

BY JOSHUA LEDERBERG AND ESTHER M. LEDERBERG[1]

THE present territorial dispute between infection and heredity might have been predicted from the bridging of the gap between microbiology and genetics. Having once learned in bacteriology that "germ" is infectious microorganism, the student finds in genetics that "germ" is hereditary constitution and the confusion persists. Our factual discussion will rely upon some recent examples from the genetics of enteric bacteria, but many authors could testify against any pretense of innovation in the theme (Darlington, 1944; Medawar, 1947; Sonneborn, 1950; Ephrussi, 1953).[2]

I. GENETIC ANALYSIS IN BACTERIA

A. Recombination mechanisms. Enteric bacteria have joined the list of model organisms for genetic research. Much of our story will concern a particular strain, K-12, of *Escherichia coli,* which was the first to be used for cross-breeding analysis in bacteria (Tatum and Lederberg, 1947; Lederberg and Tatum, 1953).

Microscopy is still an unreliable method for the detection of mating processes in *E. coli,* and we look instead for genetic signs, for cells that display new combinations of genetic traits where two parents that differ in a number of characters are grown together. It is often convenient to use genetic markers that are easily selected for or against, such as drug resistance or requirement for growth factors, for they allow recombinants to be selected at will from populations in which they are greatly outnumbered by unmated parental cells. However, highly fertile stocks are now available with which selected markers are no longer required to demonstrate recombination.

Genetic recombination can be achieved by other processes besides sexual fertilization, notably *transduction,* which is the transmission of a small fragment of genetic material from one cell to another. The physical

[1] Department of Genetics, University of Wisconsin. Paper No. 586. The experimental work cited in this paper has been supported by research grants (C-2157) from the National Cancer Institute, Public Health Service, and from the Research Committee of the Graduate School, University of Wisconsin, with funds allotted by the Wisconsin Alumni Research Foundation.

[2] The literature references in this review are not necessarily primary documentation and are chosen as most economically leading to the detailed sources.

and genetic criteria by which these mechanisms can be distinguished are summarized as follows:

Recombination Mechanisms

Mechanism	Genetic Element	Material Agency
Sex	Many linked markers (whole genome or nucleus)	Fertilization by an intact cell (gamete)
Transduction	Single marker or cluster of closely linked markers (chromosome fragment)	Transfer of subcellular (filtrable) material (DNA or virus particles)

By both criteria, recombination in *E. coli* K-12 is sexual (Lederberg, 1955). As our knowledge of these mechanisms increases, it may become advisable to refine our classification. For example, one can conceive of partial fertilizations that might be achieved by subhaploid or partially inactivated gametes (Muller and Pontecorvo, 1940; Briggs, 1952). On the other hand, Pontecorvo (1954) has proposed the term "parasexual" for any mechanism of recombination that does not partake of typical *secondary* sexual paraphernalia. The present classification adheres to nuclear fusion as the essential element of sexuality (Link, 1929), which is indeed the root of its genetic significance.

More recent microscopic studies have related recombination in K-12 to the formation of conjoined pairs of bacterial cells. The conjugants do not copulate in their entirety but eventually disjoin (Lederberg, 1956b); the relationship of conjugation to mating type is elaborated in the next section. Direct cytological evidence of karyogamy still remains to be obtained. The current controversy over the cytological definition of bacterial chromosomes will not be reviewed here; they will be postulated as the material counterparts of genetic linkage.

B. Sexual reproduction and the mating type system. The first attempts to cross K-12 (Tatum and Lederberg, 1947) were made with some trepidation. So many microorganisms have evolved mating type systems (recently reviewed by Raper, 1954; *Neurospora crassa* was the immediate example) that we feared we might have to test many strains in all combinations, a task beyond the technical resources of the time. But strain K-12 proved to be self-compatible, and the first experiment worked very

well. Many other strains have been studied since, and, in retrospect, the chances of that success were only about 0.02. Although such a figure is usually translated "statistically impossible," it is only a minor hindrance to Dr. Tatum's well-known serendipity.

That strain K-12 was self-compatible was indicated (Lederberg et al., 1951) by several facts—not only were all the original stocks derived from this strain mutually compatible, but no segregation of compatibility preferences was found among their first and second generation progeny. In hopes of finding a mating type system elsewhere, about 2,000 strains of *E. coli* that had been isolated from various sources, from turkey feces to human pus, were screened for sexual fertility by a simplified method. About 40 fertile strains had been found and were about to be tested further when a compatibility pattern emerged in K-12 itself. At the same time, we encountered an apparently sterile "mutant" and Dr. L. L. Cavalli at Milan, with whom we had been corresponding, discovered that one of the original stocks was actually self-sterile. Our collaborative studies (Cavalli, Lederberg, and Lederberg, 1953; Lederberg, Cavalli, and Lederberg, 1952) have supported the following interpretation:

The wild type strain of K-12 is self-compatible and is designated F^+. Most of the derivative stocks are likewise F^+. However, rare "mutations" for compatibility have led to the self-sterile type, F^-. Of the possible combinations, $F^- \times F^-$ is sterile, while $F^+ \times F^+$ and $F^+ \times F^-$ are progressively more fertile. A pair of standard F^+ and F^- strains can thus be used to type an unknown culture by means of the test crosses with it.

As stated before, segregation of compatibility, of F^+/F^-, had not been observed in previous crosses, although many of them had been $F^+ \times F^-$. Further experiments confirmed that $F^+ \times F^-$ crosses gave only F^+ progeny, a behavior unlike that of any other trait that has been studied in *E. coli*. The answer to this puzzle came from experiments to look for a possible hormonal stimulus from F^+ cells that might allow two otherwise incompatible F^- cultures to mate with one another. This was tested by mixing genetically labelled cultures so that different matings would give different kinds of offspring. In such mixtures, it was found that the F^- strains had been impelled to mate with each other, but this proved to be much more than a simple physiological stimulus, as the restoration of compatibility was permanent, genetically irreversible. That is to say, if an F^- strain is simply grown in contact with F^+ cells, the F^- becomes permanently F^+. The conversion is extremely efficient and occurs almost

as frequently as the F^+ and F^- cells can be calculated to collide with one another. It is therefore not surprising that the progeny of $F^+ \times F^-$ crosses become uniformly converted as if by venereal infection

The F^+ quality is extremely contagious—it will eventually spread through an entire F^- population seeded by an F^+ cell—so that it would be plausible to suppose that there is an F^+ agent or virus responsible for its spread. But repeated experiments have failed to detect any infectious particle other than the intact F^+ cell, despite the sensitivity of the test for a single particle. For example, the two mating types have been grown on opposite sides of a thin-rolled membrane filter, about 15μ thick and with pores about 1μ diameter, barely capable of holding back the bacteria. Although Dr. Grobstein, who kindly furnished the filters, has demonstrated embryonic inductions through them (1953), no passage of the F quality could be demonstrated. The F conversion in addition to its genetic quality thus appears to correspond to the contact transformations which have repeatedly been encountered in developmental studies (Weiss, 1947; Spratt, 1954; Cantino and Horenstein, 1954; Sussman and Sussman, 1956). In common with these examples, it is not known whether there is a material exchange of matrix, surface, or cytoplasm, and the expression *F^+ agent* or *particle* must be remembered as being a figure of speech.[3]

A number of mating type variations have been discovered in K-12, but cross-infection experiments have shown these to depend on the genetic constitution of the bacterium rather than on varieties of the F agent (Lederberg, unpublished; Cavalli and Ceppellini, 1953). For example, F^+ stocks vary in their apparent potency and can be gradated so that the most fertile combinations are those most widely separated. However, the conversion of a given F^- stock by contact with any one of these F^+ types results in F^+ derivatives of the same potency. Nevertheless, some evidence for variability in F among different strains, independently isolated from nature, has emerged from experiments in which K-12 F^- testers

[3] M. Delbrück, in discussing this question at the symposium, has brought to mind reactions of very high molecular order; for example, the rate of activation of phage T_4 is proportional to the 5th power of the concentration of tryptophane (Stent and Wollman, 1950). Such reactions if they occur between a cell producing the stimulus and another receiving it will display an equally abrupt dependence on the distance between the cells, and can thus account for contact transformations. However, an organized particle or patch of cell surface can be thought of as a mechanism of coordinating the elements of a high order reaction so that it will occur at a significant rate, the probability of random coincidence of the units being otherwise small. The kinetic description is not, therefore, a contradictory alternative to surface interaction theories, but it should further attempts to accomplish such transformations by high concentrations of cellular fractions, or synthetic analogues.

had been converted to F+ by the foreign strains. It has been repeatedly noticed that such F+ stocks lose their compatibility when stored for a few weeks on nutrient agar slants, in contrast to the stability of intra-strain conversions.

TABLE I. Mating types in *E. coli*.

Item	Type	Compatibility with standard F− (W-1177)	Can be "infected" [2] with F+ from K-12	Can infect standard F−
1	K-12 F−	—	+	—
2	K-12 F+	+	o	+
3	K-12 F+ aeration phenocopy	—	o	+
4	K-12 Hfr[4]	+++	—	—
5	K-12 F+ weakly compatible	±	o [1]	+
6	K-12 and others F− refractory	—	—	—
7	foreign strains F+	+	o	+ (unstable)
8	foreign strain [3] sterile F+	—	o [1]	+
9	foreign strain compatible F−	+	+	—
10	foreign strain compatible, refractory F−	+	—	—
11	most strains of *E. coli* [3] and other bacteria intersterile F−	—	—	—

[1] The compatibility reactions of these strains is unaltered by exposure to an F+ culture.

[2] The susceptibility to conversion is tested by passing the F+ quality in turn to a standard F− unless the strain is already F+, as well as by change of sexual reactivity. The symbol O indicates no test.

[3] Unlike the others, these strains are infertile in crosses with F+ as well as F− stocks.

[4] (Cavalli, 1950).

It has been suggested (Hayes, 1953) that the F agent functions in *E. coli* crosses as an extracellular vector of the gametic genes, by analogy with the role of phage in genetic transduction in *Salmonella*. In view of the abject failure of all attempts to demonstrate an extracellular agent of F conversion or recombination, this proposal is no longer current in its original form, though it may be semantically equated to fertilization by identifying the F converting agent with the F^+ cell, which is operationally correct.

The relationships between compatibility and F status have, furthermore, proved to be surprisingly complicated for an organism that was once thought to be homothallic, as illustrated by the mating types listed in Table I. These relationships show that an infective F^+ agent is neither necessary nor sufficient: the reactivity of a strain is controlled alternatively by its own constitution and by its F status. The environment also plays a role, as is shown by the aeration phenocopy: F^+ cultures can be made to simulate the reactions of F^- by cultivating them under strong aeration. These cells, however, retain the F agent and the effect is completely reversible when clones are regrown under standard conditions. At first, no method was available for the intentional production of F^- strains, and the discovery of the system had to wait upon the sporadic occurrence of two unselected mutants. P. D. Skaar (unpublished) has discovered, however, that the passage of motile F^+ strains through soft agar often results in the development of F^- variants. The mechanism of this effect, even whether it is inductive or selective, is entirely unknown, but it may be related to the continued rapid division of well dispersed bacteria at the growth frontier in this medium, by analogy with the loss of *kappa* from rapidly grown clones of *Paramecium* (Beale, 1954). At any rate, the F^- stocks obtained by this technique have been indispensable for further analyses (Nelson and Lederberg, 1954).

Hfr cultures have also been extremely useful for further studies, owing to their high frequency of recombination, and the non-contagious control of compatibility. It was first thought that Hfr $\times$ F^- crosses gave exclusively F^- progeny, but it has since been found (Cavalli and Jinks, 1956) that the two alternatives segregate, with a low frequency of Hfr, and this marker being linked to another locus called Gal, of which more is to be said.

These crosses are also the basis of recent observations of conjugation. That crosses in *E. coli* might be physiologically polarized had already been suggested by Hayes' (1953) observations on the effect of streptomycin, which, in the course of inactivating bacteria for vegetative growth,

also sterilizes F^- cells sexually. F^+ cells, however, retain some sexual reactivity. Although this was initially attributed to the extracellular persistence of postulated, extruded F^+ agents, it could equally well have been speculated that the F^- gamete contributes the bulk of the cytoplasm to the zygote while the F^+ gamete leaves behind the poisoned cytoplasm at conjugation.

That the differential effect of streptomycin is actually related to sexual differentiation is now indicated by microscopic study of Hfr $\times$ F^- conjugations. The Hfr exconjugants have given pure, unaltered clones, while the recombinants have issued exclusively from the F^- exconjugants, together with unaltered F^- cells. Fertilization thus appears to involve the passage of a nucleus from the Hfr cell to the F^- cell, wherein one fertilization nucleus or zygote is formed. The remaining nuclei of the multinucleate parental cells are unaltered, accounting for the persistence of both parental types. In sum, the process is not greatly different from hyphal fertilizations in molds, though no sexual spores have been observed. Previous studies had already demonstrated the haplobiontic life cycle with a haploid vegetative phase and a diploid zygote, which undergoes immediate reduction. If such concepts are transferable at all, it may be permissible to regard the F^- mating type as female, and the F^+ or Hfr as phenotypically determined hermaphrodites. Obligate male stocks have yet to be found, but could be detected only by attempting crosses between two males. At present, the likely point of difference between F^+ and F^- is at the bacterial surface and the ability to pair, but it may be anticipated that other steps of the sexual process are likewise subject to genetic control as in other fungi (Wheeler, 1954, and note item 8 of Table I).

The most perplexing feature of K-12 genetics is the polarized segregation so that sexual progeny tend to resemble the F^- parent more closely than the F^+ or Hfr parent. A wide variety of suggestions has been adduced to explain this fact, but they can be divided into two groups: (1) that the F^+ gamete is already defective, so that the contribution to the zygote is less from the F^+ than from the F^- side (Hayes, 1953; Wollman, 1953) and (2) that the F^+ gamete contains a full genetic complement, but that losses occur later, preferentially from the F^+ contribution to the zygote, (Nelson and Lederberg, 1954; Cavalli and Jinks, 1956; Lederberg, 1955; Cavalli, Lederberg, and Lederberg, 1953). Unfortunately, in most experiments, we can only put the parent cells together and observe the segregants that issue forth, and it is manifestly impossible to decide precisely when in the interval the evident losses have occurred.

For this reason, most of the experimental data that have been brought to bear on this problem are indecisive in so far as they deal with haploid segregants.

The fortuitous discovery (after calculated searches had failed) of unreduced, diploid sexual progeny, and of "Het" stocks that tend to generate diploids on further crossing, has led to an independent approach (Lederberg, 1949a; Nelson and Lederberg, 1954). Before the compatibility system was detected, the peculiarities of these diploids had already demonstrated that segments of chromosomes were being lost during the sexual cycle. These segments directly involve only a few markers, but a chromosome of which a piece is missing will be inviable when it stands alone in a haploid cell. Therefore, not only the deleted markers, but any loci that may be linked to them, will be affected in segregation. A detailed study of these diploids has shown (a) that the regions subject to deletion are quite invariable; (b) that the deletion always occurs; (c) that although it tends to occur on the F^+ parental chromosomes, it will sometimes occur *instead* on the F^-, from which it may be concluded (d) that the occurrence is postzygotic. To be sure, other aberrations may be postulated, too, but these are sufficient to account for the facts and to exhaust one's credulity. Furthermore, no diploids have ever been found that would correspond to the variable losses that have been proposed by other authors. Most recently, Wollman and Jacob (1955) have reported that mechanical disruption of a mating mixture at various times will influence the segregation pattern. They deduce that fertilization is normally fractional and progressive for different parts of the genome. It is not clear, however, whether the postulated fractional fertilization occurs normally or is artificially induced. Moreover, to explain the haploid segregation pattern, indirect effects on chromosome pairing and postzygotic losses could just as well be supposed. For a definite determination, it will be necessary to find unreduced partial diploids, whose occurrence and structure can be inferred only ambiguously from seeing only the final pool of segregants from many zygotes.

C. Lysogenicity and transduction. A second germinal element of *E. coli* K-12 is a bacteriophage, *lambda*. Like F, this element could be discovered only when a mutant cropped up that was an indicator for it. For twenty-five years, the bacteria had harbored this phage without revealing it to many students who had examined K-12 as a typical strain of *E. coli*. That bacteria might be lysogenic, that is maintain phage as an intracellular symbiont, had been suggested already by d'Herelle, and in the early 1930's, Burnet expressed a clear insight into the genetic im-

plications of the fact of lysogenicity. During the past five years, lysogenic systems have again attracted the interest of many virus workers (Lwoff, 1953; Luria, 1953). Burnet showed, and it was more rigorously confirmed later, that lysogenic bacteria do not contain intact virus particles; neither do sensitive bacteria soon after they have been infected with the virus. We therefore assume that the genetic element of the phage can develop in the bacterium in a latent form, the "prophage" in the lysogenic bacterium. When, under the influence of the total genotype of the infected cell, the somatic envelope is manufactured, and the virus nucleus enclosed in it, the latent phage matures into infectious particles, ready to reinitiate the parasitic cycle. In lysogenic bacteria, this transition occurs only sporadically, so that the over-all viability of the lysogenic strain is scarcely affected, but under the influence of ultraviolet light, nearly every prophage can be induced to mature, with concomitant lysis of the bacterium.

The first experimental findings on *lambda* (Lederberg, E., 1951) were (1) the parent K-12 strain and most of its descendants are lysogenic for *lambda;* (2) sensitive variants occasionally appeared among the survivors of ultraviolet irradiation (selected or induced?); (3) sensitive bacteria could be infected with *lambda,* whereupon most of the bacteria would lyse and release a new crop of the virus; however, perhaps a tenth of the bacteria gave viable clones from which lysogenic strains could be isolated.

The demonstration of lysogenicity in a sexually fertile strain opened the way to analysis of its genetic basis, that had formerly been the subject of considerable speculation. Probably most workers had an a priori conception that lysogenicity was a cytoplasmic infection (Lederberg, 1949b), analogous to the killer phenotype in *Paramecium* which depends on a factor called *kappa.* However, the results of crosses between lysogenic and sensitive strains soon showed (Lederberg and Lederberg, 1953) that lysogenicity depended on a chromosomal locus (or segment), Lp, prominently linked to another locus, Gal (for galactose fermentation).[4] The most striking bit of evidence was the synthesis of diploid heterozygotes which segregated both factors in linkage, with occasional recombinants. In addition, crosses of a *lambda*-lysogenic with another stock, carrying

[4] Some reservations with which this interpretation was first received (based on questions of F polarity, Wollman, 1953; Lwoff, 1953) appear to have been dispelled (Wollman and Jacob, 1955). They were founded not on any statistically significant discrepancies in experimental results, but upon the ambiguity of haploid segregation data already discussed. Fortunately, the behavior of heterozygous diploids answers or circumvents any questions that might pertain to the content of the original gametes.

a different phage, gave recombinants of all types, including the doubly sensitive combination. These results were incompatible with the simple cytoplasmic concept of *lambda* infection, but they do not rigorously prove that more than an indispensable component, not necessarily the entire prophage, is localized at the Lp site. Subsequent experiments by Appleyard (1953) and others have, however, shown that at least some genetic markers of the *lambda,* as well as lysogenicity itself, are localized near Gal. It is a plausible inference that the entire prophage consists of a chromosome segment of the lysogenic bacterium.

In our experience, the presence or absence of *lambda* has had no appreciable effect on segregation of Gal and other markers, unlike the decisive role of F polarity. Jacob and Wollman (1954) have, however, reported a curious interaction among their strains: when lysogenic Hfr was mated with "ly$^-$" (sensitive?) F$^-$, most of the zygotes lysed, with the production of free *lambda,* as if the prophage had been induced to mature in the course of fertilization. This effect, under their experimental conditions, may possibly be attributed to the combination of the prophage from the Hfr cell with the fresh sensitive cytoplasm of the F$^-$ conjugant, which would be comparable to the act of ordinary infection. Lysogenic × lysogenic crosses did not show the effect. The suggestion that this syngamic induction may equally well be the origin of anomalies such as segmental elimination therefore does not concord with previous studies by various workers, both on diploids and haploid segregants, which have almost uniformly involved only lysogenic parents, or with similar genetic results in sensitive × sensitive crosses.

At first, no genetic correlate of *lambda* in *E. coli* was observed. However, in the course of experiments designed to test for recombination in another enteric bacterium, *Salmonella,* a mechanism was found that was distinct from sexual fertilization. This proved to be an example of genetic transduction (defined in a previous paragraph as the transfer of a genetic fragment) in which bacteriophage particles conveyed genetic factors from the bacteria on which they were grown to new hosts (Zinder and Lederberg, 1952). Contemporaneously, the well-known pneumococcus transformation (Griffith, 1928) had begun to receive close attention in its genetic aspects and to be understood as, in effect, the historically first example of transduction, though here DNA functions directly without the benefit of a special vector. In *Salmonella,* the phage stands in for the biochemist in shattering the chromosomes of the host cell and introducing them into a recipient bacterium.

The role of phage in *Salmonella* transduction impelled a renewed study

of a possible similar role of *lambda,* in *E. coli,* notwithstanding the previous negative results. It was found (Morse, 1954, 1955; Morse, Lederberg and Lederberg, 1956) that *lambda* would transduce the Gal factor, but no others so far known. The two systems may be contrasted in several respects: (1) In *Salmonella,* nothing is known of the localization of prophage, and any genetic locus can be transduced; in K-12, the prophage is located at Lp, and only the Lp-linked factor, Gal, is transduced by *lambda;* (2) In *Salmonella,* phage is equally competent for transduction whether it is grown directly on sensitive hosts or is obtained from the ultraviolet-induced lysis of lysogenic strains; In *E. coli, lambda* is competent only when prepared by UV-induced lysis. These differences suggest that the relationship of the phage to the transduced segment is adventitious in *Salmonella,* but more direct in *E. coli.* A third difference is of another order: in *Salmonella,* transduction is promptly consummated and the transformed clone shows no residue of the segment that had been replaced; in *E. coli,* the fragment may persist indefinitely, and reproduce as such side by side with the homologous recipient chromosome.

These cells which carry an extra fragment, are called *heterogenotes.* The extra fragment itself is an *exogenote.* In heterogenotic clones, from time to time, "crossing-over" does take place between the exogenote and the intact chromosome. Whether this exchange involves physical breaks, or a modification of the replication process is no better known for transduction than for crossing-over in higher forms. The segregants may then resemble either the original recipient parent, or the strain on which the phage had been grown, or both. The persistent heterogenotes thus give insight into the intermediate stages of transduction, which proceed too rapidly for analysis in *Salmonella.*

The phage obtained from typical haploid lysogenic clones has a transduction competence of about 1 per 10^5 phage particles. By contrast, the phage obtained from heterogenotes has a competence of from 10 to 100%. That is, after correcting for virus that may have issued from segregant bacteria, virtually every phage particle from a heterogenote carries the genetic qualities of the exogenote with it. From this it can be speculated that the exogenote is the prophage itself: it is, after all, the direct descendant of a fragment that had been introduced by a previous phage infection, and which has been stringently selected for its ability to support the function of the Gal^+ gene. We may imagine that the low competence of the usual lysates reflects the unlikelihood that a random fragment will have been broken out with the right size and shape.

On this line of argument, infection and lysogenization may be considered a special case of transduction, the prophage having the dual aspect of virus nucleus and chromosome segment. Whether *lambda* originated by the mutation of a chromosome segment of an aboriginal *E. coli,* or is the reduced and integrated relic of genetic material of external origin, hybrid or parasitic, is a question in paleobacteriology that may never be answerable.

D. Gene action and position effect. In the previous discussion, Gal was referred to as a single locus controlling galactose fermentation. As seems likely to happen for any genetic locus that is studied closely enough, recurrent galactose-negative mutations have proved to be non-identical (pseudoallelic) and a series of closely linked loci can be recognized by recombination test, although the mutant phenotypes are virtually indistinguishable (E. Lederberg, 1952). In most instances, heterogenotes compounded from two distinct mutants have shown the normal galactose-positive phenotype. In some combinations, however, these heterogenotes are galactose-negative, although, having a structure $+-/-+$ (Gal_x^+ Gal_y^-/Gal_x^- Gal_y^+) they bear altogether a full complement of Gal^+ genes. Heterogenotes of the structure $++/--$ can also be synthesized, and these are galactose-positive. These combinations thus display an unmistakable *cis-trans* position effect (Lewis, 1955), that is, the two Gal^+ genes can effectively interact only in a *cis-* and not in a *trans-*arrangement, as between exogenote and chromosome (Morse, 1955).

As there are at least ten distinct loci in the Gal cluster, and probably many more, it will be interesting to look for some pattern in the position relationships, and this is currently in progress. A second type of position effect might be thought of, a possible functional distinction between genes in the chromosome as compared to the exogenote. This would amount to position effects between the loci in the exogenotic region, and loci in adjacent regions, but so far has not been detected. Position effects are the most direct manifestations of primary gene interactions and are often hypothetically explained by the interplay of nondiffusible gene products. Transduction analysis has unexpectedly led to a new approach to this fundamental problem of genetics.

Demerec (1955) has applied similar techniques to the analysis of pseudoallelic relationships in *Salmonella,* having found that mutations whose effects are biochemically related are closely linked. The startling inference that the linear sequence of these mutations corresponds to the biosynthetic sequence of reactions has also been forwarded, but the detailed numerical data in support of this inference have not yet been pub-

lished. Similar correlations between the position and function of various genes have been sought, without success, in several organisms (Cf. Sturtevant and Schultz, 1931). The examples of pseudoalleles in *E. coli* (E. Lederberg, 1952; Morse, 1955) and in fungi (Pontecorvo, 1952; Mitchell, 1955) must be distinguished from Demerec's series, as the same biochemical defect, so far is known, is associated with all the pseudoalleles in these cases. The physiological significance of such correlated sequences is even more obscure if they are unique for some processes in *Salmonella*.

When the biochemical genetics of *Neurospora* was first being developed, ca. 1940-1945, the evidence from nutritional mutants was considered to favor an elementary correspondence between single genes and single enzymes. However, semantic and experimental ambiguities have emerged (J. Lederberg, 1951; Wagner and Mitchell, 1955) and many students have now adopted a more agnostic attitude to this doctrine. Therefore, serial correlations between biochemical lesions and mutant positions (even if more than fortuitous) cannot safely be translated into an assembly line of enzyme syntheses, and might well have more to do with the functional integration of the unit steps than with the specificity of the catalysts. Whatever final interpretation is placed on these studies, they illustrate the potency for phenogenetic exploration inherent in the tools for recombination analysis in bacteria.

II. MODELS OF DEVELOPMENT

The quasi-irreversibility of differentiated clones of cells in development has posed a riddle as provoking to geneticists as it is to embryologists (Weiss, 1947). Many genetic studies with microorganisms have been motivated by their application as models of development. The genetic importance of the cytoplasm has been roundly confirmed by such studies on *Paramecium* and on yeast (Sonneborn, 1950, 1954; Ephrussi, 1953). But the corresponding de-emphasis of the developmental role of the nucleus is less warranted. These ancillary studies may help to suggest some of the possible theories that should be considered for development. Which are correct must be learned by asking the questions of embryos rather than microbes.

However *Salmonella* has furnished a probable model example of quasi-irreversible genic changes. It has been known since Andrewes' work (1922) that the flagella of this genus occur in two antigenic phases. The antigenic phase is almost stable in clonal multiplication, but an occasional cell in one phase suddenly initiates a clone of the alternative phase. The rate of transition is highly variable from one strain to an-

other, from an almost negligible frequency to as high as once per thousand cell divisions (Stocker, 1949). Altogether, some hundred different flagellar antigens are known, and occur in various combinations in different strains, but the phases of a given strain are constrained to a single pair of alternatives. This restriction already distinguishes phase variation from ordinary mutation, since we observe no mutation in antigenic specificity, only a choice of which of two alternatives will be expressed.

Transduction analysis of the flagellar antigens (Lederberg and Edwards, 1953) has confirmed that two independent loci, H_1 and H_2, control the antigenic potentialities of each strain; at each of these loci there are many alternative alleles, the combination of one H_1 and one H_2 allele defining the serological type of any strain. Thus, *Salmonella typhimurium* has the immunogenetic constitution $H_1^{\,1}\ H_2^{\,2}$, while *Salmonella abony* is $H_1^{\,b}\ H_2^{\,enx}$. Any given clone of *S. typhimurium* will, however, contain cells of either the 1- or the 2- antigenic type. What is the genetic basis of the difference between these clones of differing phase? This question can be answered in part by transduction experiments on the H_1 and H_2 loci, involving various combinations of phases of *typhimurium* and *abony*. If the phase were controlled by a cytoplasmic state, or by chromosomal factors not linked to H_1 or H_2, then the outcome of these experiments might depend on the phase of the recipient, but would be independent of the phase of the donor. In fact, the outcome does depend on the phase of both the donor and the recipient. We therefore infer that whatever element controls the phase is coupled, during transduction, with either the H_1 or the H_2 locus, or both. The details of these experiments are rather complex, and have not been fully completed or analyzed, but the H_2 locus appears to be decisive. That is, which of the two antigenic possibilities is realized seems to depend on a quasi-irreversible differentiation of the H_2 locus. The simplest speculation to rationalize the alternation of states at H_2 is that the local accumulation of the immediate products keeps this gene active, to produce more of the same, and suppresses or competes with the H_1 gene.[5]

The analysis of this genic differentiation is too flimsy, by itself, to stand as an effective model of differentiation. McClintock's (1951) work on maize has exposed an elaborate system of local genic modification, and King and Briggs (1955) have recently carried their studies of nuclear transplantation to the indisputable conclusion that the nuclei of

[5] A more detailed study which shows that phase variation depends on alternative states of the H_2 locus has now been completed (Lederberg and Iino, 1956).

developing embryonic cells in the frog are genetically altered. By further elaboration of their techniques and those of microbial genetics, it may ultimately be feasible to analyse the genetic differences among differentiated tissues no less exactly than is possible for the clones of mutant microorganisms (Lederberg, 1956a).

III. GENETIC PARTICLES

The main prop of formal genetic explanation is the "self-reproducing particle." In the last century, the problem of growth and reproduction was transposed from the whole organism to the cell, then regarded as the ultimate unit of biological structure and function. Intracellular constituents, most notoriously the genes, are now assigned the same role of the fundamental self-reproductive element of which the growth of the whole organism is the summation. But what is a "particle" and what does "*self*-reproduction" mean?

The structure of an elementary particle is a paradox the physicists have ever had to contend with. Likewise, as their instruments achieve higher resolution, biologists have had to reconcile themselves to new orders of complexity even in such atomistic units as genes. In genetics, "particle" is used in two senses: an abstract inference from breeding data and a microscopic object. Unfortunately, the correspondence of the formal and material units has rarely, if ever, been proven.

A. Formal particles. The geneticist usually infers a particle from discontinuities in inheritance: segregation in sexual progeny, mutations, unequal cell divisions. Thus Mendel was able to deduce the basic laws of diploid inheritance from the results of crosses with peas without considering the material nature of his Anlagen. Mutational discontinuities, independent for different qualities, led many students to adopt a particulate theory of inheritance in bacteria before this could be confirmed by recombination technique (Luria, 1947; Lederberg, 1948). In yeast (Ephrussi, 1953; Spiegelman, 1951) particles have been inferred to explain discontinuities in the transmission of a trait in vegetative reproduction, after the example of Sonneborn and Preer with *Paramecium* (Beale, 1954). The evaluation of target number and size in radiobiological experiments has been a fashionable exercise, whether or not the implied targets had any independent standing as real biological units, leading sometimes to absurd conclusions (Lea, 1947). The outstanding success of Mendelian analysis has possibly blunted the general criticism that these particles are only formal descriptions of cell division, a mathematical simplification that leaves untouched the question of their materiality.

This criticism is most realistic for particles that have not been directly visualized or for which inconsistencies have emerged. For example, Ephrussi's study (1953) of the induction of respiratory-deficient mutants, among the buds of yeast cells exposed to acriflavine, has implied that little of the cytoplasm of the mother cell is passed to the bud. From a technically analogous study of another trait, adaptation to galactose, Spiegelman (1951) inferred an equipartition of the maternal cytoplasm.

Unequal cell divisions generally (including stem cells, indeed the primary act of differentiation) have been and are apt subjects of a formal particle analysis, but primarily to suggest material hypotheses that can be independently checked. For a microbial example, in experiments on the transduction of motility to nonmotile strains of *Salmonella* (Stocker, Zinder, and Lederberg, 1953; Lederberg and Stocker, 1955), cells that had acquired the motile phenotype could be isolated and followed directly under the microscope. Only a small fraction of these isolates generated simple motile clones, corresponding to the previous knowledge of transduction of other markers. Most of the initial motile cells gave clones in which motility was transmitted to a limited number of cells, from 1 to 100. These motile cells in turn generated unbranched chains of descent. At each successive fission of the motile cell in such a hereditary chain, one motile and one nonmotile daughter were produced, a segregation so sharp that it was at first thought to be certain evidence of the persistence of a nonreproducing particle. How 1 to 100 such particles would be generated in one clone will be discussed elsewhere. We may still question here whether the bald description of unequal division is not more informative than the postulation of a motility-conferring particle. The so-called particle might simply be the mathematical representation of a rule of cell division, that the locomotive machinery is not randomly divided, or if it divides at all, the lesser parts are incompetent. The particle hypothesis does lead to certain lines of inquiry (e.g. whether chain cells are uniflagellate), but we must also remember how little we know of the mechanics of cell division as it pertains to cytoplasmic structure in any organism.

Unequal division is a regular feature of the vegetative growth of certain diatoms: the cell wall consists of two rigid half-walls, one fitting inside the other like the halves of a Petri dish. At cell division, the half-walls separate, and a new half-wall is secreted *within* each. The previous inner half, which is the smaller, thus serves as the outer half-wall at the next generation, and this cell is therefore smaller than its sister. The average cell size of a clone thus becomes progressively smaller, but may

be restored through the sexual auxospore stage (Wiedling, 1948). The morphological description of cell division saves us from a rather elaborate particle formulation which might otherwise be invoked to account for the size classes and progressive diminution in a diatom culture. Alternatively, we are reminded that the formal "particle" does not only imply a material granule, but a polarized process at cell division.

Numerical data on the partition of cellular organelles that might bolster the usual hypothesis of random partition are unfortunately very scanty. Wilson (1931) has recorded some figures on the partition of the 24 chondriospheres in the spermatocyte to the spermatids, in a scorpion. The partition is inexact, but although Wilson quoted it as random, the distribution is actually much more compact.

Another interpretation of particles is in the chemical terms of a steady state, alternative chains of reactions being assumed to compete with one another (as suggested, for example, by Delbrück; see Beale, 1954). This mode of formal description can apply, among others, to real particles too, the mathematical laws of competition being relevant even to populations of free living organisms. This formulation is therefore not, as has been erroneously suggested, an alternative hypothesis to particles, but a more general, and possibly more fruitful mode of description (Pollock, 1953). Efforts to find experimental discriminations are therefore likely to be foredoomed by tautology.

B. Visible particles. (For documentation of the following section, see J. Lederberg, 1952.) At least since Altmann's bioblasts, various particles that have been seen within cells have been imbued with genetic functions. Unfortunately, the imputation has rarely been backed by critical proof. Three kinds of inclusion may be considered: the chromosomes, mitochondria and other organelles, and endosymbionts. The last two are distinguished in principle by the postulated identity of the latter with independent organisms, but as this is a matter of techniques and definition, all extra-chromosomal particles that function in heredity may be classified together as plasmids.

That the chromosomes are the material sites of the formal genes is no longer disputed. There is no question of the exact correspondence between the two constructs throughout the life cycle of many organisms and under the most exhaustive experimental stresses. To anticipate the following discussion of plasmids, can we, however, postulate a third invisible element of which the chromosomes and the formal genes in their linear groups are both subordinate manifestations? In a sense, we do if we postulate, as many authors have, that only a part of the chromosome

has genetic functions, the remainder being inessential to its basic continuity. If there is a third element, it would have to be the invariable companion of the chromosomes everywhere, e.g. in the compact sperm head; the most dedicated critics of the chromosome theory have exhausted themselves in efforts to separate the ideal from the real chromosomes.

The genetic quality of the plasmids is much more doubtful. The most convincing correspondences apply to those plasmids that can be cultivated outside their usual hosts, such as the rickettsial symbionts of arthropods or the yeast-like symbionts of beetles, though these have generally been remembered for their pathogenic or nutritional rather than genetic functions. For other plasmids, formal particles have been inferred on other evidence, but the correspondence rests on very shaky evidence, if any. The problem is not very different from that of the etiology of infectious disease, which in the early development of bacteriology, lay in chaos before Robert Koch had presented his famous four postulates: (1) The microbe must invariably accompany the disease. (2) It must be isolated from the diseased tissue and grown in pure culture. (3) The pure culture must reproduce the disease when reinoculated in healthy animals. (4) The same microbe must be reisolated from the artificial infection. If plasmid is substituted for microbe, and phenotype for disease, the applicability of these postulates in genetics is obvious. For present day technology, the second criterion may be too stringent, and chemical purification may be substituted for pure culture. In that event, the validity of the proof will rest on the reliability of the purification, and on indirect evidence that the particle can grow *in vivo*.

Kappa in *Paramecium* perhaps best illustrates the use of these criteria (Beale, 1954). The number and size of (formal) *kappa* particles was estimated before they fed the hope that *kappa* could be visualized. By microscopic observation, particles were then discovered which satisfied all but the second criterion, as cross-infection was accomplished only by conjugation, not from pure culture. Logically (if not very plausibly), it can still be argued that the true genetic element is undiscovered, and that *kappa* (like paramecin in turn) is an epiphenomenon. More recently, it has been possible to transmit *kappa* efficiently by cell-free homogenates, which if sufficiently purified may serve for the second criterion and complete the proof (Sonneborn, personal communication).

Other plasmids, especially the chloroplasts, have been identified by less secure inferences. In many plants, breeding tests have shown that the presence or quality of chloroplasts may be maternally, presumably cyto-

plasmically, controlled. The chloroplasts are the most prominent inclusion in the cytoplasm, but there is no other evidence that they are the genetic element in question. An analogous statement applies to the respiratory granules in yeast (Ephrussi, 1953). Since chloroplasts can be removed from many plants by treatment with streptomycin, there is an attractive opportunity to try reinfection experiments which have not hitherto been reported. The mitochondria are still less certain, as cells that have been deprived of their mitochondria are not likely to be viable. However, Lettré has stated that devitalized ascites tumor cells that had been cytolysed in distilled water could be resuscitated by artificial reinfection with suspensions of granules. Unfortunately, the experiments could not be regularly reproduced, and it is too soon to ask further questions, whether the granules are "self-reproductive elements" or repair the damaged cells only in a physiological sense. Where viability is the sole phenotypic effect, such a distinction may be difficult, but one can study the specificity as to source of the reparative material. Another approach is suggested by LeClerc's experiments (1954) on enzymatic stimulation in chick chorioallantois treated with liver microsomes. Her data do not, however, show whether the granules have multiplied.

C. Self-reproduction. Living and self-reproducing (SR) are probably synonymous concepts, though one is often used to explain the other; the semantics (meaninglessness) of "life" and "living" has been ably exposed by Pirie (1937). The prefix "self" is the stumbling block to useful understanding, since to detect reproduction (without self) is often only a problem in arithmetic. SR must be interpreted as some degree of self-sufficiency, but with respect to what? If SR is to have any material meaning, it must apply at least to whole organisms, and these we know are dependent on the outside world at least for the energy and substance of reproduction.

SR might, as the next resort, mean self-sufficiency in information or specificity, but again the least exacting autotrophic organism must obtain its substance in specific chemical forms, and has a negligible probability of survival if randomly situated in the universe. A great deal of information is inherent in the specifications of the terrestrial biosphere. We are accustomed to discounting these nutritional problems, and might consider an entity as (almost) SR if it proliferates on a medium whose chemical composition is (almost) definable. Alternatively, self-sufficiency is, at best, a relative concept, the inverse of the least specificity required of the environment. To date, chromosomes and most plasmids cannot be cultivated *in vitro,* and we know nothing of their demands on the host

cytoplasm, whether they are of such a different order from the nutrition (information input) of more familiar microorganisms to warrant a unique classification on other than technical grounds.

If their self-sufficiency is only relative, chromosomes (and plasmids) are assuredly self-necessary or self-dependent, that is to say the cell lacking a chromosome also lacks at least part of the information to produce it again. The experimental criteria of infection or disinfection to prove self-dependence of plasmids have already been discussed, but even if these are satisfied we still cannot assess the degree of autonomy, the relative information inherent in the particle and in the cell, and this remains unknown even for the chromosomes. (There are recurrent hints of cytoplasmic control of genic specificity, Sonneborn, 1954; Michaelis, 1954.) Before we can discuss this question meaningfully, we shall have to learn and adopt a plausible measure of biologically significant complexity. (Cf. the nature-nurture controversy, Hogben, 1951.)

Self-dependence is not only relative to the other-dependence on the cell, but is inherently a statistical concept that should not be used too rigidly. Any configuration of matter is statistically possible: a self-dependent particle may be thought to improve the chances of its own recurrence, but this probability is measured neither 0 without, nor 1 with, the particle. In a complex biological system, the independent emergence of a particle or reaction system might be called a mutation from less differentiated substance, as has happened throughout evolution as well as in controlled experiments, (Spiegelman, 1951; Pollock, 1953).

Self-dependent replication can be attributed to systems that are trivial models of living organisms, because the parts and the product are either both simple or both complex. Crystallization of complex salts and the autocatalytic conversion of trypsinogen to trypsin are examples often quoted; in a universe of IBM machines, a punched card would be a self-reproducing particle, too. If trypsin resynthesized itself from amino acids rather than trypsinogen, we would be more impressed. What is distinctive about organic reproduction, aside from its chemical rather than electromechanical workings, is the gap between the simplicity of the parts and the complexity of the product. Of this, the previously stated criteria for SR tell nothing. Mutability has been suggested as a further criterion, but this is also reducible to a measure of complexity: a particle with only two components that could be independently lost already can manifest four alternative states, one of them null. The full meaning of organic SR, short of its complete description, can perhaps

be developed only in terms of a knowledge of the nutritional input to the particle.

In considering the origin of life, and the possibility of constructing useful models, many authors have postulated the unique, sudden, and improbable creation of a complex living molecule. This *macro*mutational hypothesis is contrary to the contemporary trend of thought which relies upon the concatenation of innumerable, more probable but less ambitious steps to account for further evolutionary development. If the distinction of organic SR is complexity, we might suppose that simple inorganic systems are potential starting points for organic evolution though few have progressed far enough to be recognized as living. The application of binary coding in computing machines illustrates how the most complex information may be expressed as an array of the simplest constituents. In searching for working models of SR, the means by which the *autocatalytic units* can be chemically coordinated to form *SR complexes* may be the most urgent basis of choice of any one among many autocatalytic processes.

BIBLIOGRAPHY

Andrewes, F. W. 1922. Studies in group-agglutination. I. The *Salmonella* group and its antigenic structure. *J. Path. Bact. 28,* 505-521.

Appleyard, R. K. 1953. Segregation of *lambda* lysogenicity during bacterial recombination in *E. coli* K-12. *Cold Spr. Harb. Symp. 18,* 95-97.

Beale, G. H. 1954. *The Genetics of Paramecium Aurelia.* Cambridge Univ. Press, London.

Briggs, R. 1952. An analysis of the inactivation of the frog sperm nucleus by toluidine blue. *J. Gen. Physiol. 35,* 761-780.

Cantino, E. C., and E. A. Horenstein. 1954. Cytoplasmic exchange without gametic copulation in the water mold *Blastocladiella emersonii. Amer. Natur. 88,* 143-154.

Cavalli Sforza, L. L. 1950. La sessualità nei batteri. *Boll. Ist. sieroter. Milan. 29,* 281-289.

Cavalli Sforza, L. L., and R. Ceppellini. 1953. Unicità del Fattore F+ in *E. coli* K-12. *Notizario di Diagnostica e Terapia. 27,* 3-13.

Cavalli Sforza, L. L., and J. L. Jinks. 1956. Studies on the genetic system of *E. coli* K-12. *Jour. Genet. 54,* 87-112.

Cavalli, L. L., J. Lederberg, and E. M. Lederberg. 1953. An infective factor controlling sex compatibility in *Bacterium coli. J. Gen. Microbiol. 8,* 89-103.

Darlington, C. D. 1944. Heredity, development and infection. *Nature 154,* 164-168.

Demerec, M. 1955. What is a gene?—twenty years later. *Amer. Natur. 89,* 5-20.

Ephrussi, B. 1953. *Nucleo-cytoplasmic relations in micro-organisms.* Oxford Univ. Press, Oxford.

Griffith, F. 1928. The significance of pneumococcal types. *J. Hyg. 27,* 113-159.

Grobstein, C. 1953. Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. *Nature 172,* 869.

Hayes, W. 1953. The mechanism of genetic recombination in *Escherichia coli. Cold Spr. Harb. Symp. 18,* 75-93.

Hogben, L. 1951. The formal logic of the nature nurture issue. *Acta genetica statistica medica. 2,* 101-140.

Jacob, F., and E. Wollman. 1954. Induction spontanée du développement du bactériophage λ au cours de la recombinaison génétique, chez *Escherichia coli* K-12. *C. R. Acad. Sci. Paris. 239,* 317-319.

King, T. J., and R. Briggs. 1955. Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. *Proc. Nat. Acad. Sci. U.S. 41,* 321-325.

Lea, D. E. 1947. *Actions of radiations on living cells.* MacMillan, New York.

LeClerc, J. 1954. Etude de la glucose-6-phosphatase dans la membrane chorioallantoïdienne traitée par les microsomes du foie. *Experientia 10,* 251.

Lederberg, E. M. 1951. Lysogenicity in *E. coli* K-12. *Genetics 36,* 560.

Lederberg, E. M. 1952. Allelic relationships and reverse mutations in *Escherichia coli. Genetics 37,* 469-483.

Lederberg, E. M., and J. Lederberg. 1953. Genetic studies of lysogenicity in *Escherichia coli. Genetics 38,* 51-64.

Lederberg, J. 1948. Problems in microbial genetics. *Heredity 2,* 145-198.

Lederberg, J. 1949a. Aberrant heterozygotes in *Escherichia coli. Proc. Nat. Acad. Sci. U.S. 35,* 178-184.

Lederberg, J. 1949b. Bacterial variation. *Ann. Rev. Microbiol.* 1-22.

Lederberg, J. 1951. Genetic studies with bacteria. In *Genetics in the 20th Century,* edited by L. C. Dunn. Macmillan, New York.

Lederberg, J. 1952. Cell genetics and hereditary symbiosis. *Physiol. Rev. 32,* 403-430.

Lederberg, J. 1955. Recombination mechanisms in bacteria. *J. Cell. Comp. Physiol. 45 (Suppl. 2),* 75-107.

Lederberg, J. 1956a. Prospects for a genetics of somatic and tumor cells. *Ann. N.Y. Acad. Sci. 63,* 662-665.

Lederberg, J. 1956b. Conjugal pairing in *Escherichia coli. J. Bact. 71,* 497-498.

Lederberg, J., L. L. Cavalli, and E. M. Lederberg. 1952. Sex compatibility in *Escherichia coli. Genetics 37,* 720-730.

Lederberg, J., and P. R. Edwards. 1953. Serotypic recombination in *Salmonella. Jour. Immunol. 71,* 232-240.

Lederberg, J., and T. Iino. 1956. Phase variation in Salmonella. *Genetics.* In press.

Lederberg, J., E. M. Lederberg, N. D. Zinder, and E. R. Lively. 1951. Recombination analysis of bacterial heredity. *Cold Spr. Harb. Symp. 16,* 413-443.

Lederberg, J., and B. A. D. Stocker. 1955. "Phenotypic" transductions of motility in *Salmonella. Genetics 40,* 581.

Lederberg, J., and E. L. Tatum. 1953. Sex in bacteria: genetic studies, 1945-1952. *Science 118,* 169-175.

Lewis, E. B. 1955. Some aspects of position pseudoallelism. *Amer. Natur. 89,* 73-89.

Link, G. K. K. 1929. Reproduction in thallophytes, with special reference to fungi. *Bot. Gaz. 88,* 1-37.

Luria, S. E. 1947. Recent advances in bacterial genetics. *Bact. Rev. 11,* 1-40.

Luria, S. E. 1953. *General Virology.* Wiley, New York.

Lwoff, A. 1953. Lysogeny. *Bact. Rev. 17,* 269-337.

McClintock, B. 1951. Chromosome organization and genic expression. *Cold Spr. Harb. Symp. 16,* 13-47.

Medawar, P. B. 1947. Cellular inheritance and transformation. *Biol. Rev. 22,* 360-389.

Michaelis, P. 1954. Cytoplasmic inheritance in *Epilobium* and its theoretical significance. *Advances in Genetics 6,* 287-401.

Mitchell, M. B. 1955. Aberrant recombination of pyridoxine mutants of *Neurospora. Proc. Nat. Acad. Sci. U.S., 41,* 215-220.

Morse, M. L. 1954. Transduction of certain loci in *Escherichia coli* K-12. *Genetics 39,* 984-985.

Morse, M. L. 1955. Cis-trans position effect in transduction heterogenotes of *Escherichia coli. Genetics 40,* 586-587.

Morse, M. L., E. M. Lederberg, and J. Lederberg. 1956. Transduction in *Escherichia coli. Genetics. 41,* 142-156.

Muller, H. J., and G. Pontecorvo. 1940. Artificial mixing of incompatible germ plasms in *Drosophila. Science 92,* 418, 476.

Nelson, T. C., and J. Lederberg. 1954. Postzygotic elimination of genetic factors in *Escherichia coli. Proc. Nat. Acad. Sci. U.S. 40,* 415-419.

Pirie, N. W. 1937. The meaninglessness of the terms life and living. In *Perspectives in Biochemistry,* edited by J. Needham and D. E. Green. Cambridge Univ. Press, London.

Pollock, M. R. 1953. Stages in enzyme adaptation. In *Symp. on Bacterial Adaptation.* Soc. for Gen. Microbiol., Cambridge.

Pontecorvo, G. 1952. Genetical analysis of cell organization. *Symp. Soc. Exp. Biol. 6,* 218-229.

Pontecorvo, G. 1954. Mitotic recombination in the genetic systems of filamentous fungi. *Proc. 9th Int. Cong. Genetics.* I. 192-200.

Raper, J. R. 1954. Life cycles, sexuality and sexual mechanisms in the fungi. In *Sex in Microorganisms,* edited by D. H. Weinrich. Pp. 42-81. A.A.A.S., Washington, D.C.

Sonneborn, T. M. 1950. The cytoplasm in heredity. *Heredity 4,* 11-36.

Sonneborn, T. M. 1954. Patterns of nucleocytoplasmic integration in *Paramecium*. *Proc. 9th Int. Cong. Genetics. I.* 307-325.

Spiegelman, S. 1951. The particulate transmission of enzyme-forming capacity in yeast. *Cold Spr. Harb. Symp. 16,* 87-98.

Spratt, N. T., Jr. 1954. Physiological mechanisms in development. *Physiol. Rev. 34,* 1-24.

Stent, G. S., and E. L. Wollman. 1950. Studies on activation of T_4 bacteriophage by cofactor. II. The mechanism of activation. *Bioch. Bioph. Acta 6,* 307-316.

Stocker, B. A. D. 1949. Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhi-murium*. *J. Hyg. 47,* 398-413.

Stocker, B. A. D., N. D. Zinder, and J. Lederberg. 1953. Transduction of flagellar characters in *Salmonella*. *J. Gen. Microbiol. 9,* 410-433.

Sturtevant, A. H., and J. Schultz. 1931. The inadequacy of the sub-gene hypothesis of the nature of the scute allelomorphs of *Drosophila*. *Proc. Nat. Acad. Sci. U.S. 17,* 265-270.

Sussman, M., and R. Sussman. 1956. Cellular interactions during the development of the cellular slime molds. This symposium. Pp. 125-154.

Tatum, E. L., and J. Lederberg. 1947. Gene recombination in the bacterium *Escherichia coli*. *J. Bact. 53,* 673-684.

Wagner, R. P., and H. K. Mitchell. 1955. *Genetics and metabolism.* Wiley, New York.

Weiss, P. 1947. The problem of specificity in growth and development. *Yale J. Biol. Med. 19,* 235-278.

Wheeler, H. E. 1954. Genetics and evolution of heterothallism in *Glomerella*. *Phytopathology 44,* 342-345.

Wiedling, S. 1948. Beitrage zur Kenntnis der vegetativen Vermehrung der Diatomeen. *Botaniska Notiser,* pp. 322-354.

Wilson, E. B. 1931. The distribution of sperm-forming materials in scorpions. *J. Morph. 52,* 429-483.

Wollman, E. 1953. Sur le déterminisme génétique de la lysogénie. *Ann. Inst. Pasteur. 84,* 281-293.

Wollman, E., and F. Jacob. 1955. Sur le mécanisme du transfert de matériel génétique au cours de la récombinaison chez *Escherichia coli* K-12. *C. R. Acad. Sci. Paris. 240,* 2449.

Zinder, N. D., and J. Lederberg. 1952. Genetic exchange in *Salmonella*. *J. Bact. 64,* 679-699.

VI. CELLULAR INTERACTIONS DURING THE DEVELOPMENT OF THE CELLULAR SLIME MOLDS

BY MAURICE SUSSMAN AND RAQUEL R. SUSSMAN [1]

I. INTRODUCTION

To the microbiologist, development is essentially a foreign concept. His unit of experimentation is the individual cell rather than an organized, multicellular entity. Phenotypic divergence in representative microbial populations is considered to be populational variation, not development. When, however, he encounters protistal forms which surpass their phylogenetic associations by a display of capacities that to his intuitive sense are developmental, he is forced to sit under the Bo tree and contemplate the problem of distinguishing between populational variation and development.

His trouble stems from the fact that both systems possess common properties. Thus, in microbial populations, one can observe that the emergence of phenotypic heterogeneity is strictly ordered in physiological time, and that the proportions of the cell types are precisely controlled through selective or inductive effects of the environment and by interactions between the cells. One such example is the rough-smooth variation of *Brucella abortus* (Braun et al, 1951). As the smooth inoculum grows it excretes alanine to which the smooth variety is sensitive but not the rough. A progressive selection of the latter type ensues so that it dominates the population during the stationary growth phase. The proportions of the rough and smooth cells are regulated by the concentration of alanine. Another example is the "long-term" adaptation of the yeast *Saccharomyces chevalieri* to galactose utilization (Spiegelman et al, 1950). Exposure to galactose induces a small number of cells to ferment the sugar rapidly and to transmit this capacity to their progeny. The exact proportion of the colonies displaying the positive phenotype is determined by interactions between adapted and unadapted cells. In these cases, intuition tells the microbiologist that he is not dealing with developmental events.

[1] Northwestern University, Evanston, Illinois. The experimental work was accomplished with the aid of grants from the Office of Naval Research and the National Institutes of Health.

A necessary condition to developmental systems is that there be a transient or permanent association of the component cells in compact, multicellular aggregates. One could very well define development and populational variation operationally according to the presence or absence of this condition,[2] and having done so, slam the conceptual door between them to study each in comfortable isolation. But this teaches us nothing about the logic behind this distinction and so lends no insight into development.

Leaving the door slightly ajar however, one may ask what multicellularity contributes to so complicate and refine cell variation as to force the introduction of a new name. Multicellularity provides many opportunities but perhaps most significant is the scope that it lends to cellular interactions in determining pathways of differentiation and morphogenesis and in controlling their extents. In particular, the fact that the interactions can proceed under geometric constraints enables them to attain enormous complexity and specificity. For without spatial separation, the indiscriminate commingling of many chemical messengers would surely provoke a chaos of effects. Further, the apposition of effector and responder cells can provide the system with the opportunity to use direct cell-cell contact as a regular mode of interaction. This in turn allows the use of nondiffusible or highly labile substances as purveyors of stimuli and thereby extends the versatility of the system and the sensitivity of response.

Perhaps then, only a quantitative difference exists between the populational variation of a microbial culture and the development of a chick embryo. It may be that the same elements present in the first system are greatly complicated and extended through the existence of multicellularity in the second so that we are led to establish a qualitative distinction between them. In any case, it is clear that investigation of the genetic and physiological consequences of cellular interactions must serve as a major approach to the definition and understanding of development. For this reason we shall devote the remainder of this discussion to several cases of cellular interaction that occur during the development of the cellular slime molds. It should be noted that the subject matter will be restricted to cases uncovered by work in our laboratory, thus omitting several of the most important and potentially

[2] This definition does not necessarily exclude the complex Protozoa from the sphere of developmental study. They may be considered *acellular* counterparts of simple metazoa and metaphytes; an alternate pathway to attainment of the advantages inherent in a high level of somatic organization.

valuable ones indicated by the studies of K. B. Raper (1940a, b) and J. T. Bonner (1950, 51).

II. THE DEVELOPMENTAL CYCLE

Detailed descriptions of the developmental cycle of *Dictyostelium discoideum* (Raper) and related species have been provided by Olive (1902), Arndt (1937), Raper (1941, 1952), and Bonner (1944).

The individual cells are called myxamoebae and are morphologically and ecologically comparable with true protozoan soil amoebae (Singh, 1947a, b). They feed upon bacteria (Raper, 1937, 1939) and proliferate by binary fission. In the laboratory they may also be cultivated upon a defined medium containing two vitamins, a purine-pyrimidine mixture, salts, and a homogenous protein fraction obtained from gram negative bacteria (Bradley and Sussman, 1952; Sussman and Bradley, 1954). At the stationary growth phase, they come together in multicellular aggregates in response to the production of specific attractive substances generically termed "acrasin" (Bonner, 1947). Each conical aggregate is then transformed into a cigar shaped pseudoplasmodium which in at least two species (of which *D. discoideum* is one) migrates over the agar surface through considerable distances. Culmination then follows wherein the pseudoplasmodium undergoes morphogenetic alterations to produce in *D. discoideum* a sessile fruit with a spore mass at the top, a cellulose sheathed parenchymatous stalk, and a basal disc below. The form and pigmentation of the fruit varies with different genera and species. The spores are ultimately disseminated and, under the proper conditions, discard their cellulose jackets to germinate into vegetative myxamoebae. A schematic diagram of the cycle is given in Fig. 1.

III. SYNERGISTIC AND ANTAGONISTIC INTERACTIONS BETWEEN MORPHOGENETICALLY DEFICIENT MUTANTS

When spores or myxamoebae are delivered in low number on an agar plate and spread with a few drops of *Aerobacter aerogenes* culture, plaques appear within the area of bacterial growth after three days (Sussman, 1951). These plaques represent clones of myxamoebae and the cells within them can subsequently aggregate and culminate to produce normal fruits. If the inoculum is irradiated with ultraviolet so as to obtain a kill of 95 to 99%, a small proportion of the clones that appear display aberrant development. These aberrations are generally heritable and have provided us with a large number of stable,

developmental mutants (Sussman and Sussman, 1953). Some of the mutants can complete the cycle and form fruits but these are bizarre in appearance. Figures 1-3 on Plate I show wild type *D. mucoroides* and two such mutants. Others are morphogenetically deficient in that they cannot complete the developmental sequence but instead stop at intermediate stages. Some of them have been classed as "aggregateless" mutants, those that cannot aggregate at all, remaining as dispersed

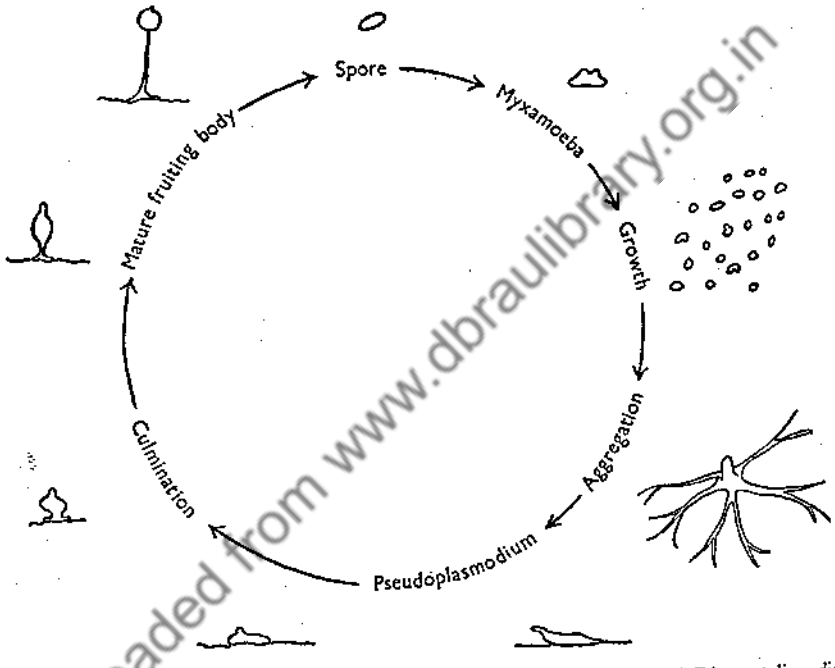


Fig. 1. A schematic representation of the developmental cycle of *Dictyostelium discoideum Raper* (Sussman, 1954).

amoebae on growth plates and "fruitless," those that can aggregate and thereafter stop development short of fruit formation. Plate II shows the wild type and a fruitless mutant of *D. discoideum*.

A study has been made of the developmental capacities of 18 deficient stocks both when maintained alone and when mixed in paired combinations (Sussman, 1954). With regard to the first condition, Fig. 2 shows schematically the levels of development attained by clones on growth medium and by cells that had been washed free of bacteria and dispensed on washed agar-distilled water plates (minimal medium). No cell proliferation occurs on such plates. The stocks fell into four general classes:

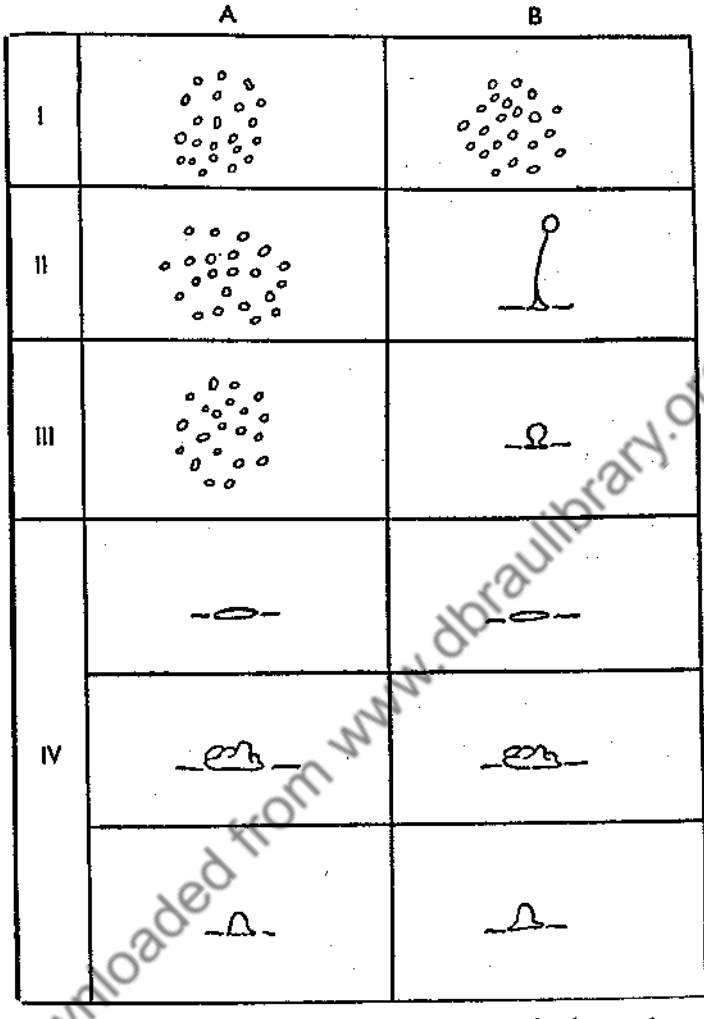


Fig. 2. The extents of development in the four phenotypic classes of morphogenetically deficient mutants on (A) growth medium and (B) minimal agar (Sussman, 1954).

I. Aggregateless on growth medium, aggregateless on minimal.
II. Aggregateless on growth medium, wild type on minimal.
III. Aggregateless on growth medium, fruitless on minimal.
IV. Fruitless on growth medium, fruitless on minimal.

Table I shows the disposition of the 18 stocks among the four phenotypic classes. It should be noted that the stocks of class II breed true after fruiting on minimal agar. Obviously their development is inhibited by something in the growth medium or the bacteria.

TABLE I. Disposition of the morphogenetically deficient strains among the phenotypic classes (Sussman, 1954).

I	II	III	IV
Agg-53	Agg-55	Fr-4	Fr-1
53A	58	Fr-5	Fr-2
57	200	Fr-7	Fr-3
59	205		Fr-6
204	208		
206			

Washed cell suspension of strains from classes I, III, and IV, were mixed and placed on minimal agar. Many of the pairs could develop significantly further together than when alone and could produce mature fruits with viable spores. Fig. 3 is a schematic summary of the types of synergistic development observed. Some pairs produced normal

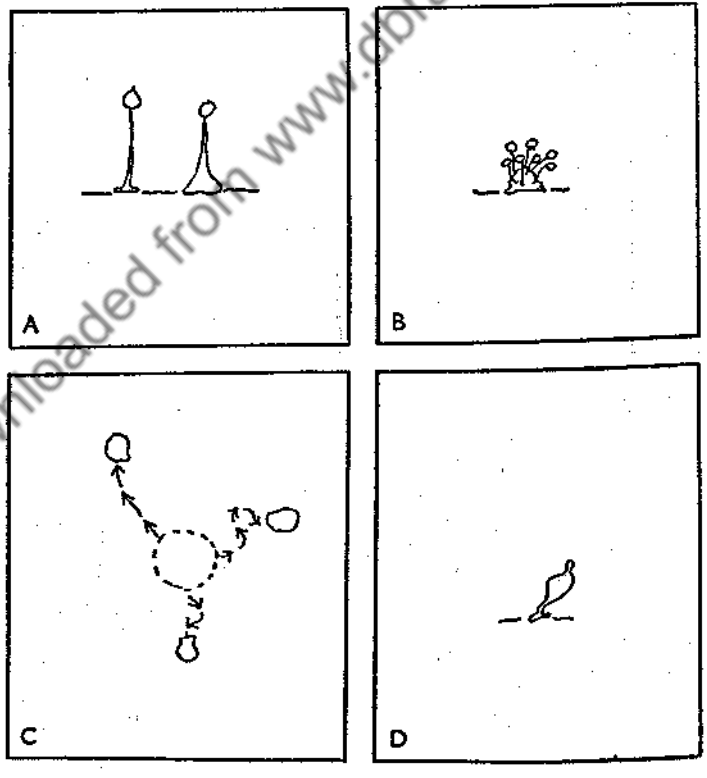


Fig. 3. Types of synergistic development (Sussman, 1954)

or thick-stemmed fruits (type A). Others produced "bushy fruits" (type B). The latter occurred in cases where one of the deficient partners had been derived from the bushy mutant, Bu-1, of *D. discoideum*. This mutant produces a fruit just like that of type B. Thus the deficient strains derived from Bu-1 carry the genetic potentiality for constructing bushy fruits although the expression of that potentiality is limited to occasions where synergism is possible. The third type of development was restricted to pairs that included strain Fr-1. Alone this strain produced a flat, loose aggregate. When mixed with certain other deficient stocks, the aggregates proceeded to migrate over the agar surface much like pseudoplasmodia but failed to fruit at the end of their travels. Finally, one pair developed synergistically, but not to completion (type D).

TABLE II. The dependence of synergistic development on the proportions of the cell types. Strain Fr-1 combined with Agg-53 (Sussman, 1954).*

Strain		Mature fruits	Flat migrating aggregates
Fr-1 (No. cells $\times 10^{-4}$)	Agg-53		
100	1	0	0
100	10	0	++
100	50	++	+++
100	100	+++	+++
50	100	+++	+++
10	100	+++	0
1	100	++	0

*Comparative number and size of fruits and flat migrating aggregates scored by number of + signs.

The type and intensity of the development depended upon the proportions of the two partners in the original mixture. Table II summarizes the data for mixtures of Fr-1 and Agg-53. This pair produced flat, migrating aggregates that did not fruit and pseudoplasmodia that formed normal fruits. When one partner was in great excess only the former appeared. When the other partner was in great excess only the latter appeared. Both types of development could be produced by intermediate ratios.

Table III summarizes the comparative response patterns of six aggregateless and seven fruitless strains when mixed in all possible combina-

tions. It may be noted that Agg-53, 53a, and probably Agg-57 (which was lost during the course of the experiments) showed identical patterns in that they all responded synergistically with Fr-1 and Fr-3 but with no others, nor was there intragroup synergism. Similarly, Agg-59 and 204 showed identical patterns (despite the fact that Agg-59 was derived from the wild type and Agg-204 from the bushy variant). The response of Agg-206 was different from the others. All seven fruitless stocks gave independent patterns. This would imply that there are at least three independent metabolic defects that can produce the aggregateless condition and at least seven that lead to fruitlessness.

TABLE III. The comparative synergistic response patterns of the deficient stocks (Sussman, 1954).*

	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6	Fr-7	53	53A	57	59	204	206
Fr-1		—	—	X	—	X	X	X	X	X	X	X	—
Fr-2	—		—	—	—	—	—	—	—	—	X	X	—
Fr-3	—	—		—	X	X	X	X	X	X	X	X	X
Fr-4	X	—	—		X	—	X	—	—		—	—	—
Fr-5	—	—	X	X		—	—	—	—		—	—	—
Fr-6	X	—	X	—	—		—	—	—		—	—	—
Fr-7	X	—	X	X	—	—		—	—		—	—	—
53	X	—	X	—	—	—	—		—	—	—	—	—
53A	X	—	X	—	—	—	—	—		—	—	—	—
57	X	—	X	—	—	—	—	—	—		—	—	—
59	X	X	X	—	—	—	—	—	—	—		—	—
204	X	X	X	—	—	—	—	—	—	—	—		—
206	—	—	X	—	—	—	—	—	—	—	—	—	

*X, synergistic combination; —, non-synergistic combination.

Two other items are of interest here. One is that no two aggregateless strains were found to synergize in paired combination. Subsequent investigation to be described later indicated that although all of the aggregateless stocks can react to acrasin (the chemotatic agent involved in aggregation), none of them can produce it. Evidently they cannot, when paired, exchange metabolites so as to overcome each other's deficiencies in acrasin production.

The second item is the failure of many pairs, whether fruitless or aggregateless, to synergize even though they had independent response patterns. Ideally, one would expect that only those pairs having identi-

cal biochemical deficencies would fail to respond to one another, but that any heterologous pair would respond. The fact that this does not occur suggests either that the test system is not sensitive enough to detect faint synergistic capabilities or else that these deficiencies lead to side reactions and the appearance of metabolites in kind or quantity able to interfere with synergism.

A number of attempts have been made to distinguish among the possible mechanisms of synergistic development and the field has been narrowed considerably. Four mechanisms were originally proposed:

1. Syngamy or heterokaryosis between deficient partners to produce genetically competent individuals.
2. The exchange of diffusible metabolites to shunt the biochemical blocks responsible for the deficiencies.
3. The exchange of nondiffusible substances by direct cell contact.
4. The contribution by the partners of a complete array of cell types needed for normal development but without exchanges of material other than those that would normally exist (i.e. acrasin production).

The first of these has been made unlikely by the fact that an intensive search for recombinant types or mixed clones arising from synergistic developments has not been successful. Table IV shows the results of spore analyses of three synergistic pairs. Out of 4,085 clones examined, only the parental types were encountered. Incidentally, it is of interest that the proportions of the partners within the spore assemblies were determined by the nature of the pair and by the ratios of cells in the original mixture. Subsequently, R. R. Sussman (results unpublished) carried out a more thorough search for recombinants and sampled not only the spores but also cells from all stages of the synergistic developments without success. The possibility does exist that transient heterokaryons formed and broke up before development was completed. However, if such a process affected an appreciable number of cells, it would have been detected.

The second possibility has been subjected to as crucial a test as we could envisage (Sussman and Lee, 1955). Preliminary experiments using a variety of approaches failed to demonstrate that synergism proceeded through the exchange of diffusible materials. However, the objection could be raised in each case that the diffusible substances might be highly labile and that the test system used could not afford extensive enough contact between the partners to permit synergism. In order to provide intimate and continuous contact between the partners

over a large area the deficient strains were dispensed on both sides of very thin agar membranes. The membranes had been prepared by dipping a stainless steel cylinder flanged at one end (Plate I, 4) into molten agar. Upon removal, a film of agar adhered to the flanged end. After cells were dispensed on the agar surfaces the assemblies were incubated in sealed jars in a water saturated atmosphere.

TABLE IV. Phenotypic analysis of spores from synergistic fruits (Sussman, 1954).*

Combination	No. cells × 10^{-4}		Number clones examined	Number aggless clones	% aggless clones
Fr-1 × Agg-204	100	100	778	5	0.6
	50	50	1137	11	1.0
	50	100	126	1	0.8
Fr-2 × Agg-59	50	50	271	12	4
	50	25	203	4	2
	50	10	443	0	0
Fr-2 × Agg-204	50	100	582	45	8
	10	100	86	36	42
	5	100	459	308	67

* Spores from synergistic fruits were collected and plated on growth medium with bacteria. The resulting clones were then scored. Only clones bearing the parental phenotypes were encountered.

With one trivial exception, the mutants when opposed by other deficient strains or the wild type, developed precisely as they did alone. This, despite the fact that the average membrane thicknesses were about 30μ or 2 to 3 cell diameters. To answer the objection that highly labile substances might not penetrate the membranes thin as they were, it was shown that acrasin, which has a biological half-life of the order of one minute at room temperature, (B. M. Shaffer, private communication) could penetrate as much as 200μ of agar thickness. This was detected by a study of the coincidence of wild type and fruitless mutant aggregative centers and streams above and below the membranes. Thus it is probable that for the mutant stocks now at hand, synergistic development is not mediated by exchange of diffusible metabolites.

Another observation of interest was that aggregateless cells, opposed by the wild type of fruitless variants across a membrane, tended to

cluster opposite aggregative centers and streams. This confirmed the earlier finding (Sussman, 1952) that these strains can respond to the aggregative stimulus imposed in mixtures of wild type and mutant cells. The fact that the mutant myxamoebae cannot then form their own aggregate indicates that though sensitive to acrasin, they cannot produce it.

The third and fourth explanations have not been fully evaluated. The latter (no material exchange) seems aesthetically unattractive but unfortunately can be attacked only by devious arguments. One of these is raised by examination of the synergistic mixture, Fr-1 × Agg-204. When alone, Fr-1 aggregates but produces no spores. Yet when mixed with Agg-204, it supplies at least 99% of the spore population (Table IV). It is difficult to see how the sporogenesis of Fr-1 could proceed except by passage of material from Agg-204. Difficult, but not impossible; and so the argument is indicative rather than crucial.

The possibility of exchange by direct contact emerges as the most likely mechanism and also most provocative of interesting experiments. A few techniques have been envisaged whereby cytoplasmic exchange might be demonstrated if such occurs. One that we anticipate attempting in the near future is to feed each partner on small, nondigestible, and differently colored particles in the manner used by Metchnikoff in his classical demonstration of phagocytosis. If retained by the myxamoebae, these would then serve as indicators of cytoplasmic exchange during synergistic development or during the normal developmental sequence for that matter.

It is equally important to extend this study to a larger collection of deficient stocks. Perhaps some can be found that synergize in a specific manner by exchange of diffusible agents thus providing an easier entrance into the intermediary metabolism of the developmental stages.

The investigation of the morphogenetically deficient mutants also revealed an example of a specific antagonistic interaction. Agg-208, obtained from the bushy variant, is a stock that falls into category II. That is, clones on growth medium are aggregateless but the washed cells can aggregate and fruit on minimal agar. Spores taken from such fruits retain the aggregateless phenotype when plated on growth medium. Agg-206, also derived from Bu-1, is aggregateless under any conditions but will synergize in mixtures with Fr-3. When mixtures of washed Agg-208 and -206 were placed on minimal agar, the latter inhibited the aggregation of the former. This inhibition was much more intense than the nonspecific effect of growth medium on Agg-208 aggre-

gation since a ratio of as little as 1 : 10 of 206 to 208 was inhibitory. The specificity of this interaction was shown by the failure of Agg-206 to inhibit the aggregation of Agg-58, a stock whose morphogenetic capacities paralleled those of Agg-208. Further, no aggregateless stock excepting Agg-206 could inhibit Agg-208.

Preliminary experiments have indicated that the inhibitory capacity of Agg-206 can be dissociated from viability by freezing and thawing of these cells, prior to mixing.

IV. CELLULAR INTERACTIONS DURING AGGREGATION

The aggregation stage of slime mold development has been described in detail elsewhere (Raper, 1941; Bonner, 1944, 1947). Its main features include: (a) a sudden and dramatic elongation of the cells accompanied by enhanced pseudopodial activity; (b) radial orientation of the cells and a concerted migration toward the centers of orientation; (c) formation of radial, branched streams by the incoming amoebae; and (d) the appearance of a conical aggregate of cells at the center. The size of the aggregate, length of the streams, and branching patterns differ among the species (Raper, 1951).

Several examples of cellular interaction have been observed during aggregation. Bonner in an elegant demonstration of chemotaxis (1947, 1949) showed that the cells of *D. discoideum* are attracted to the aggregative centers by a specific diffusible substance which he termed acrasin. Studies of mixed aggregations (Raper, 1940a; Raper and Thom, 1941) and of the effects of active material leached from aggregates and pseudoplasmodia upon heterologous myxamoebae (Shaffer, 1953) indicate that the material is at least genus specific. Acrasin is produced not only at the aggregative center but along the cell streams (Bonner, 1947). Recent experiments of Shaffer (private communication) suggest that the cells are first sensitized and oriented by the concentration gradient of acrasin and then start producing acrasin themselves.

Another example of interaction is shown in the relationship between the number of aggregative centers that may be formed by a population of constant size and the population density (Sussman and Noel, 1952). Fig. 4 illustrates this relation in *D. discoideum*. Constant numbers of washed myxamoebae were dispensed in drops of different volume on washed agar. The area covered by a drop being a function of the volume delivered, control over population density could be achieved. Subsequently, the fluid was absorbed by the agar and the cells were found to be homogenously distributed within the confines of each drop. After

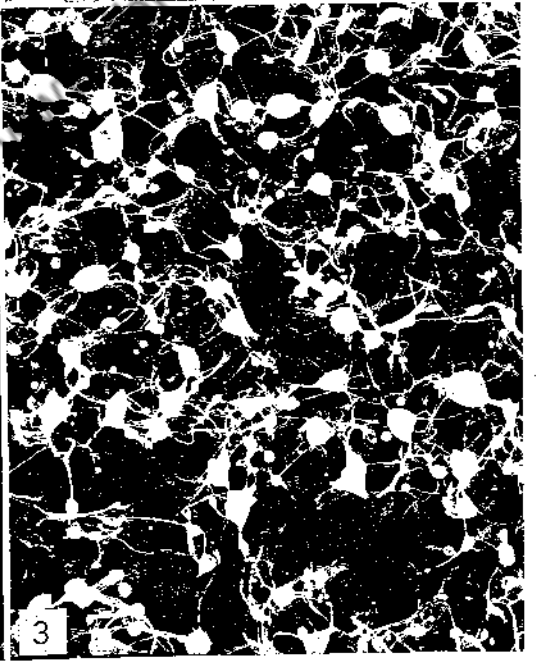


PLATE I. 1. The glassy mutant of *Dictyostelium mucoroides*. 2. The forked mutant of *D. mucoroides*. 3. Wild type *D. mucoroides* fruits (Figs. 1-3 from Sussman and Sussman, 1953). 4. The cylinder used for making thin agar membranes (Sussman and Lee, 1955). After preparation of the membrane, the cylinder was incubated flanged end upward, leaving space for cells on either side of the membrane to construct fruits.

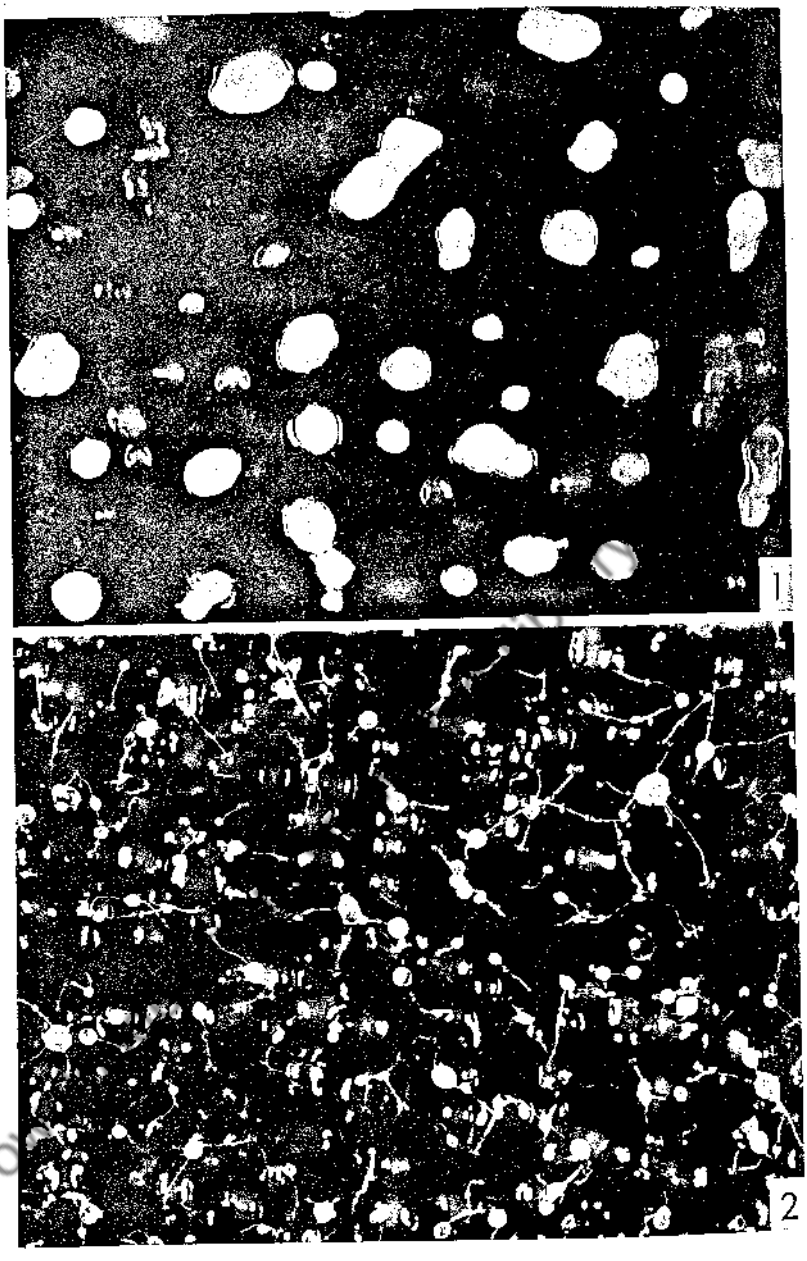


PLATE II. The terminal state of the fruitless mutant Fr-1. 2. The wild type *Dictyostelium discoideum* fruits. (Sussman and Sussman, 1953.)

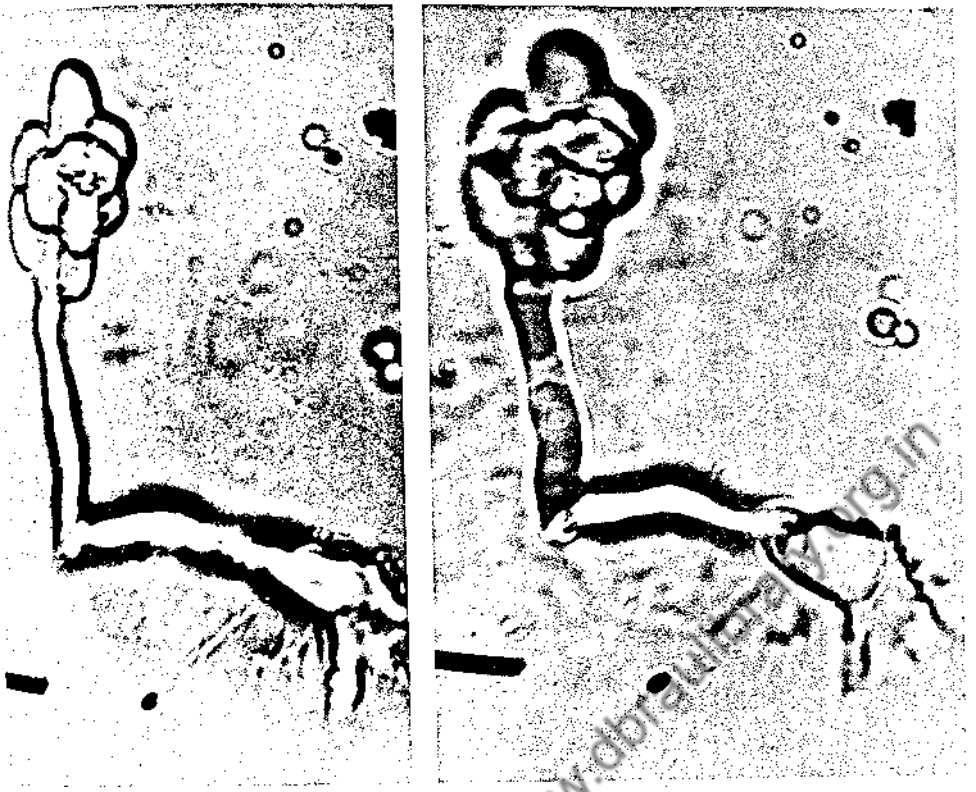


PLATE III. Fig. 1. Photomicrographs taken at two focal planes of an Fty-1 fruit in situ (Sussman, 1955).

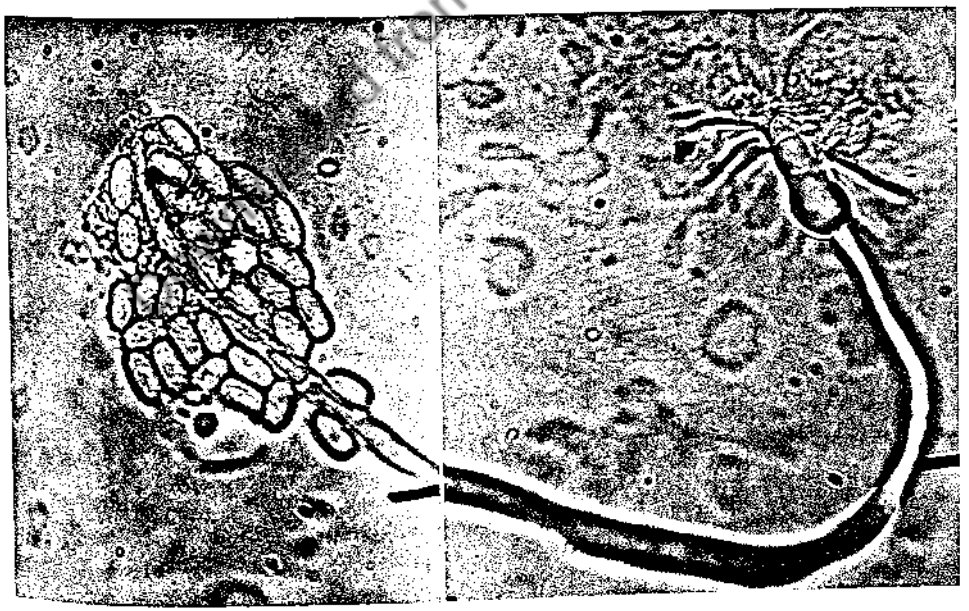


PLATE III. Fig. 2. A photomicrograph in situ of an Fty-1 fruit (Sussman, 1955). ×950.

16 to 24 hours' incubation, the numbers of aggregates or fruits (one from each aggregate) were determined. In Fig. 4 curves for three population sizes (1×10^5, 5×10^4, and 2.5×10^4 cells) are shown. At population densities that were very low (below 80 cells per mm^2), the cells could not respond to the aggregation stimulus. At high densities, all the cells aggregated but a submaximal number of centers was formed. It may be that the close proximity of potential center-forming agencies

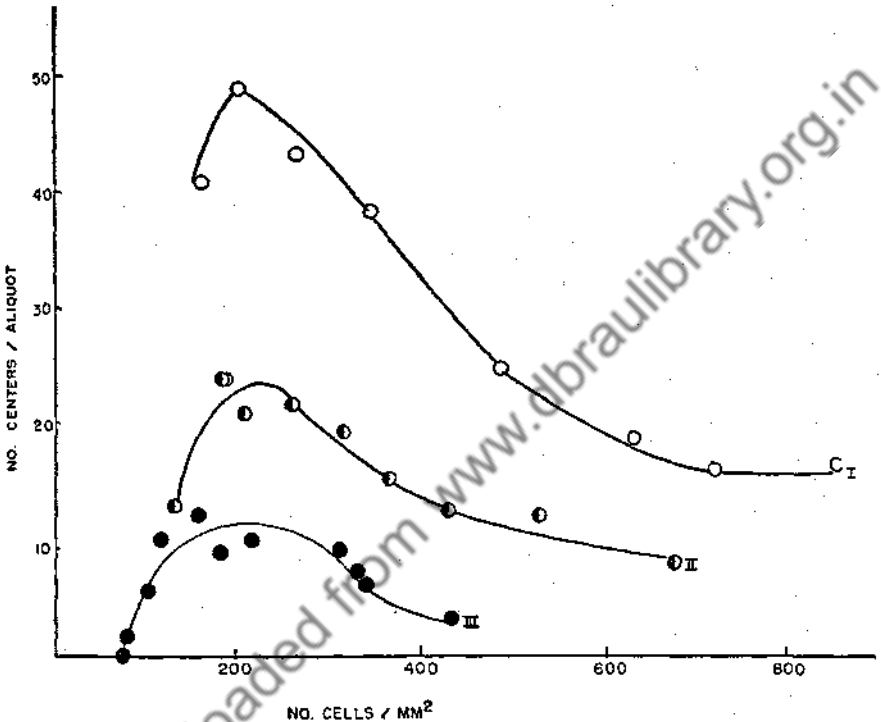


Fig. 4. Center formation in *Dictyostelium discoideum* as a function of population density and cell numbers (Sussman and Noel, 1952).

results in competition between them for the same responder cells. Thus, only the winners in this competition form centers. Alternatively, one might imagine that the high density of responder cells exerts an inhibitory effect upon the potential center-forming agencies and eliminates some. At a density of 200 cells/mm^2, the maximum number of centers was formed, proportional to the number of cells present. As seen, in *D. discoideum* a distribution of approximately one center for every 2200 cells was obtained under these conditions. The optimal density for center formation and the number of centers that appear are char-

acteristic of the species. In *D. purpureum,* approximately one center is formed for every 300 cells present and the optimal density is in this case about 100 cells/mm². In general, the performance of *D. discoideum* during aggregation is influenced to only a slight extent by changes in the environment. Most physical or chemical alterations that are not lethal leave the relations between center formation and cell numbers or population density untouched. In an extensive survey (Bradley and Sussman, 1955), only histidine and adenine and its relatives were found to produce significant effects. The first enormously increased the sensitivity of the cells and permitted aggregation to proceed at exceedingly low densities. The second made the cells highly insensitive and greatly increased the optimal density.

A variety of independent experimental approaches has indicated that the proportionality between the number of aggregative centers and the number of cells results from the presence in the population of a small number of "initiator cells," each of which evokes the formation of a center by its neighboring "responder" cells. Further, the proportion of cells that act as initiators while constant for any one system, depends upon the inherent sensitivity of the responder cells present and the distance over which the initiative stimulus must travel.

TABLE V. Populational distribution of center forming capacity (Sussman and Noel, 1952).*

Number of cells/drop	Number of drops examined	Proportion of drops containing:				Ratio of centers to cells
		0 centers	1 center	2 centers	3 centers	
2100	85	0.42	0.50	0.07	0.012	1:2400
1025	91	0.63	0.32	0.044	0.011	1:2200
900	123	0.69	0.29	0.025	0.0	1:2400

* Very small replicate samples of washed cells were dispensed on minimal agar. After incubation, the proportions of samples containing 0, 1, 2, 3 centers were determined. The ratio of centers formed to cells present was calculated by means of the Poisson series.

Very small replicate samples of washed *D. discoideum* myxamoebae were dispensed on washed agar at high population density (Sussmann and Noel, 1952). Samples of 1000 to 2000 cells were employed. After incubation the distribution of samples with no centers, or 1, 2, or 3 centers was determined. Table V shows the results of three experiments. The fact that a large number of samples contained no centers

and that all centers appeared before 24 hours and none after, indicates that only a small proportion of cells could initiate aggregation and that these were distributed by chance among the samples. The inference of random distribution was confirmed by an analysis of variance between the observed fractions of samples with 0, 1, 2, and 3 centers and the theoretical Poisson distribution. The average number of centers per sample could be calculated from the first expression of the Poisson series, $P_0 = e^{-m}$ where P_0 is the proportion of samples with no centers, e, the naperian base and m the average number of centers per sample. The ratios of centers formed to cells present could be calculated from these values and the known number of cells per sample, and they are given in the last column of Table V. They are in close agreement with the ratios obtained from the curves in Fig. 4.

Another series of experiments employed mixtures of *D. discoideum* wild type and aggregateless mutants that could not aggregate spontaneously but would respond to aggregative stimuli imposed by the wild type cells (Sussman, 1952). Replicate 0.01 cc drops were dispensed on washed agar containing a constant number of wild type and varying numbers of aggregateless myxamoebae. In different experiments the numbers of wild type per drop ranged from 2000 to 8000: few enough so that, alone, they were too dispersed to form any centers at all at the low extreme or more than one or two at the high extreme. The addition of aggregateless cells made it possible for centers to appear. A progressive increase in the number of centers coincided with increases in the number of aggregateless cells until a plateau was reached. Fig. 5 shows the data from three mixtures of wild type with varying numbers of Agg-53. It may be seen that the plateau values were characteristic of the number of wild type present in the mixture.

In the presence of excess aggregateless cells, a change in the number of wild type would affect only the number of initiator cells, not the quantity of responders. Under these conditions, were a center initiated by more than one wild type cell, the number of centers would vary exponentially with the number of wild type cells present. In contrast, if a center were evoked by a single wild type myxamoeba, the number of centers should vary linearly with the number of wild type. Fig. 6 shows this relationship and it is seen to be linear. Curve 1 was obtained in mixtures with excess Agg-53 and curve 2 with Agg-53A, another strain. These results therefore buttress the conclusion that single initiator cells in the wild type population evoke the formation of aggregative centers.

The question arises as to what is the precise proportion of initiator to responder cells in the population. Thus far, we have had the confusing experience of obtaining almost as many different answers as the kinds of experiments that we do. Using the wild type alone as the test system, a ratio of 1 : 2200 was obtained. In mixtures with Agg-53A the ratio of wild type initiators to responders was 1 : 1720 and with Agg-53, 1 : 980. Recent experiments with other and more sensitive aggregateless stocks have provided wild type ratios as low as 1 : 250.

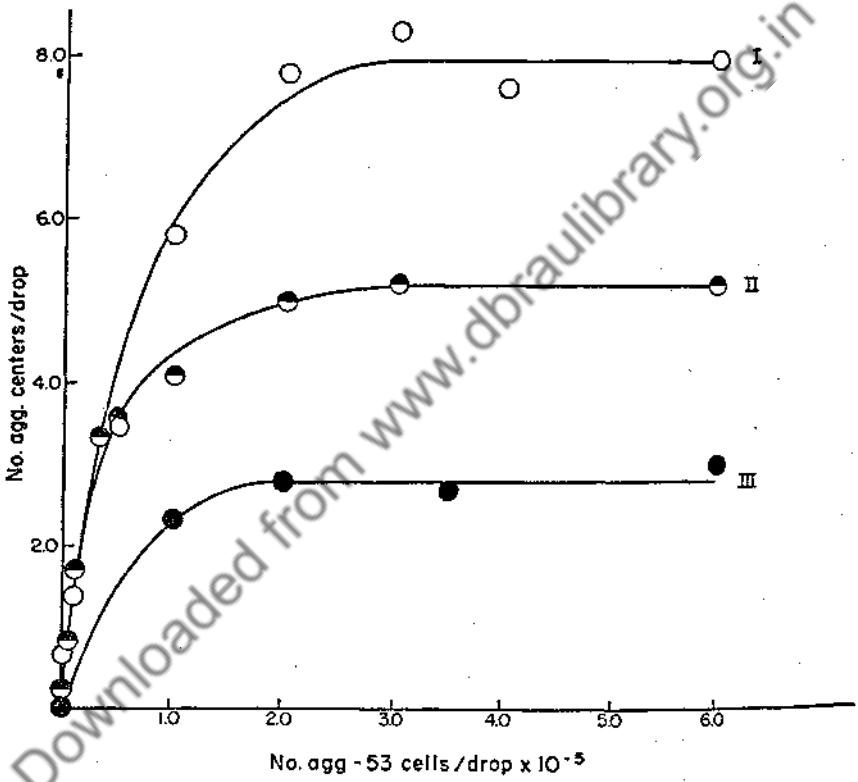


Fig. 5. The relation between the number of centers formed and the number of Agg-53 present in mixtures with Wild Type NC-4. Curve I was obtained for mixtures containing 8,000 wild type and varying numbers of Agg-53, curve II with 5,000 wild type, and curve III with 2,500 (Sussman, 1952).

At least two explanations can be provided to account for the discrepancies observed through using a variety of aggregateless stocks in the test system. One is that each aggregateless strain in mixtures with wild type responds to a different set of "initiators." Thus, Agg-53 finds that one peculiar variety of wild type cell can satisfy its requirement

for an aggregative stimulus and this variety is present in the ratio of 1:980. In contrast, Agg-53a requires a different and rarer variety of wild type cell present in the ratio of 1:1720. The trouble with the explanation is the fact that since each aggregateless stock seems to provide us with a new ratio, a point may be reached where there will be more wild type varieties than wild type cells. A second and more likely possibility is that the aggregateless stocks respond to different propor-

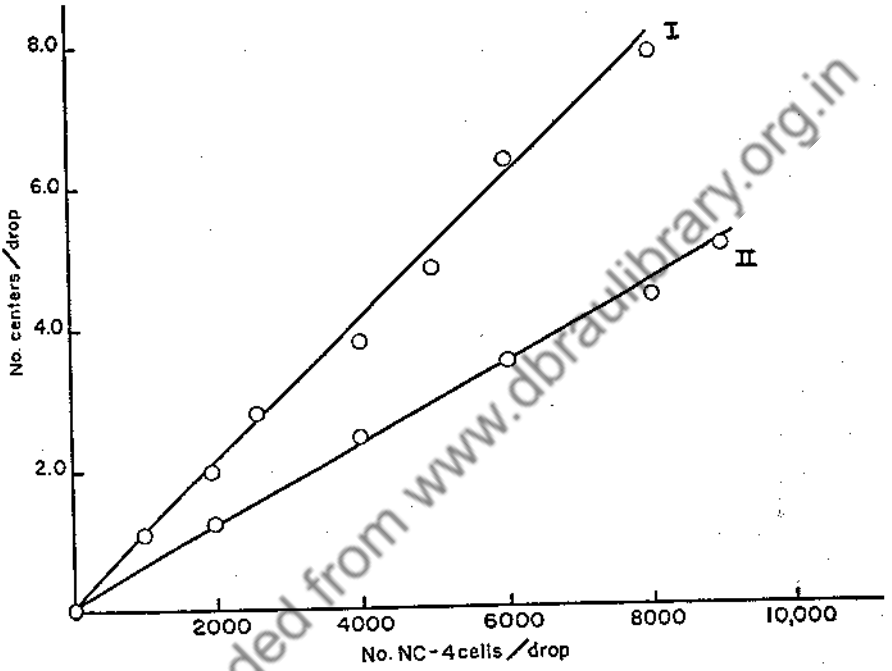


Fig. 6. Formation of aggregative centers by mixtures of Wild Type NC-4 with excess numbers of: I, NC-4 Agg-53 (number of centers/cell = 1.02×10^{-3}); II, NC-4 Agg-53A (number of centers/cell = 5.8×10^{-4}). (Sussman, 1952.)

tions of the same class of initiator cells. If the latter displayed a spectrum of initiative capacity, the conditions of experiment and the inherent sensitivity of the responder cells would determine what proportions of the wild type could be scored as initiators. In this way the discrepancies observed could be ascribed to differences in the sensitivity of the aggregateless cells to the initiative stimulus. This postulate implies that in the wild type population the difference between initiator cells and responders is not genic but reflects random or induced physiological variation against a constant genic background.

This interpretation is supported, albeit not crucially so, by unpublished data concerning the aggregative performance of wild type cells taken from various stages of the growth cycle. Were the emergence of the initiator cells the result of persistent genetic alteration, one might expect that the ratio of initiator to responder cells at the stationary growth phase would have been determined by the comparative selective pressures for or against the two types during exponential growth. Consequently in the course of the growth cycle, starting with spores and ending at the stationary phase, the ratio of initiators to responders should be subject to systematic changes as a result of the selection. However, the aggregative performances of newly germinated spores, myxamoebae at the end of the Lag phase and populations taken from different parts of the Log phase, when tested on washed agar, proved to be identical within the limits of experimental error.

It seems more likely that there are no initiator and responder cells per se during growth but that the cessation of growth provides conditions that induce some cells to attain initiative capacity while others must perforce be responders, much in the same way that the presence of galactose induces an adaptive transformation in a few cells in a culture of *Saccharomyces chevalieri*.

Two alternative biochemical roles have been ascribed to the initiator cell (Sussman, 1955a). It may be merely a super acrasin producer that can begin acrasin synthesis spontaneously and then sensitize its neighbors. Alternatively, the initiator might produce a co-factor distinct from acrasin but necessary to its synthesis. At present there is no evidence to confirm or reject either possibility.

V. THE DEVELOPMENT OF THE FRUITY MUTANT OF D. DISCOIDEUM

A mutant was recently isolated from UV-treated *D. discoideum* whose study has shed additional light upon both aggregation and the later phases of development (Sussman, 1955b). The mutant was noticed because of its ability to produce a huge number of proportionately tiny fruits on growth plates and was accordingly christened "fruity" and designated Fty-1.

The aggregative performance of washed Fty-1 cells on minimal agar is shown in Fig. 7. It differs from that of the wild type (Fig. 4) in several conspicuous ways. Most evident is the much greater number of centers produced by the mutant. At the optimal density, 10,000 mutant myxamoebae formed 423 centers (See Table VI) for a ratio of centers

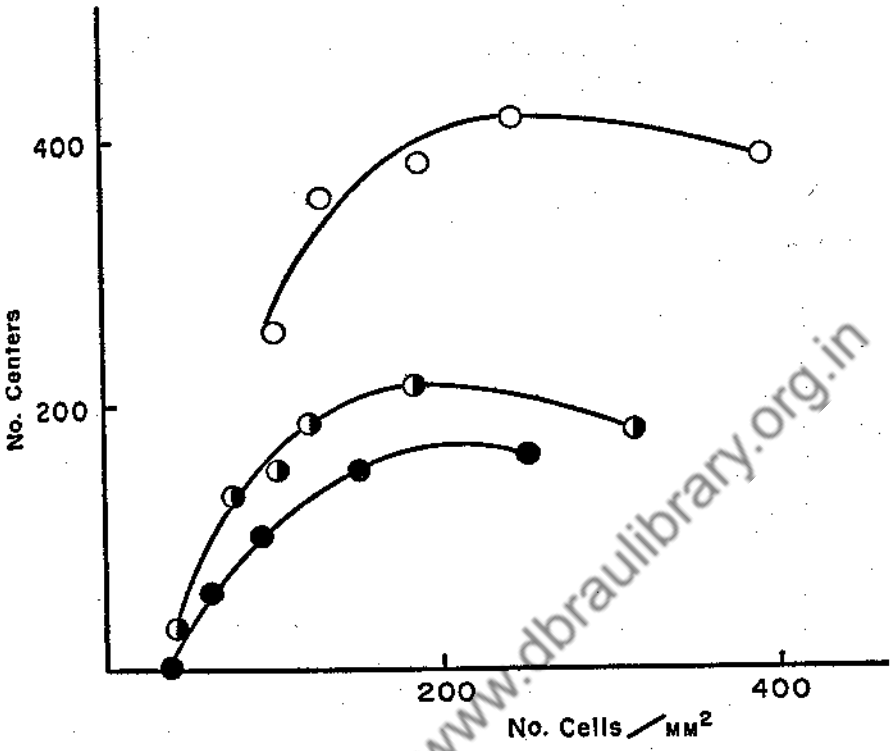


Fig. 7. Relation between center formation by Fty-1 and population density (Sussman, 1955). The upper curve was obtained with samples of 10,000 cells, the middle with 5,000, and the lower with 4,000.

to cells of 1 : 24 whereas the same number of wild type produced about 5. In addition, the threshold density for mutant aggregation was about 40 cells/mm² to be compared with 80 for the wild type. Finally the depression of center formation at high densities was only half as intense in the mutant as in the parental stock.

TABLE VI. The relation between center formation and cell number at the optimal density for strain Fty-1 (Sussman, 1955).

Number of cells	Number of centers	Ratio centers : cells	Optimal density
10,000	423	1 : 24	240
6,000	250	1 : 23	180
5,000	213	1 : 24	200
4,000	168	1 : 24	210

The tremendous increase in aggregative centers was imagined to arise in at least two ways. If the mutant responder cells were exceedingly sensitive to the initiative stimulus, then many putative initiator cells, too weak to attract the wild type responders, might be effective in exciting the mutants. Alternatively, it could be supposed that the genetic change from wild type to fruity produced an absolute increase

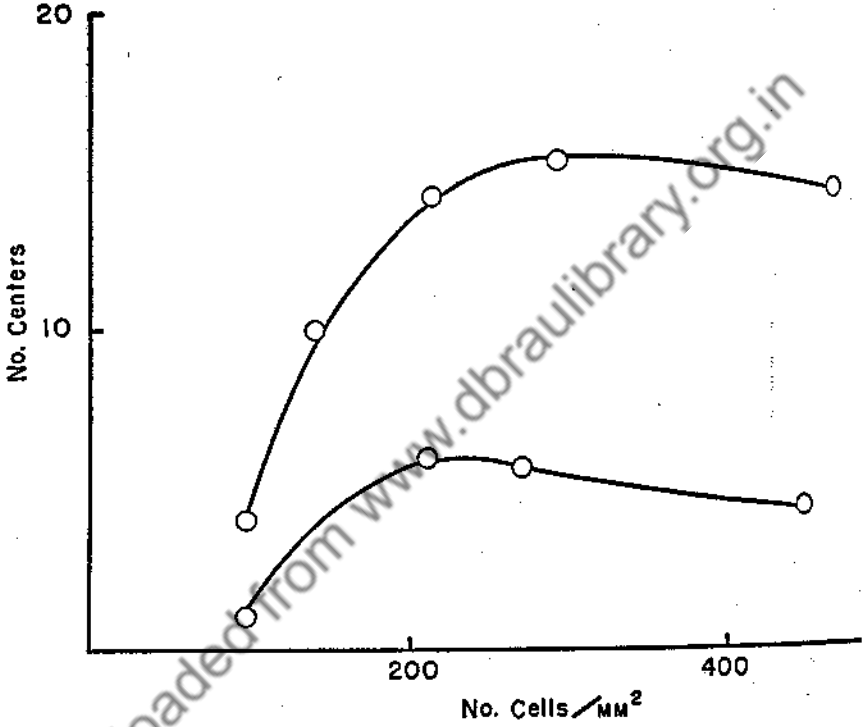


Fig. 8. Center formation by mixtures of *Dictyostelium discoideum* wild type and Fty-1. The upper curve was obtained with samples containing 500 Fty-1 and 10,000 wild type at different population densities; the lower with 10,000 wild type alone (Sussman, 1955).

in the number of initiator cells to which any responder, whether wild type or mutant, could react. These postulates were tested by a study of the aggregation of mixtures of wild type and mutant myxamoebae. The mixtures contained a very large proportion of wild type cells to supply the responder element and a small enough number of mutant cells so that their contribution to the assembly if any could only be a supply of initiators. Under these conditions a significant increase in center formation by the mixture over that formed by the wild type alone would demonstrate that the mutant population did indeed con-

tain more initiator cells than the wild type and, furthermore, that the former were capable of attracting wild type as well as mutant responders.

Fig. 8 shows the data from one determination out of the 9 performed. Samples containing 10,000 wild type and 500 Fty-1 cells were dispensed over a range of population densities (upper curve). The lower curve shows the data for the same number of wild type without addition of

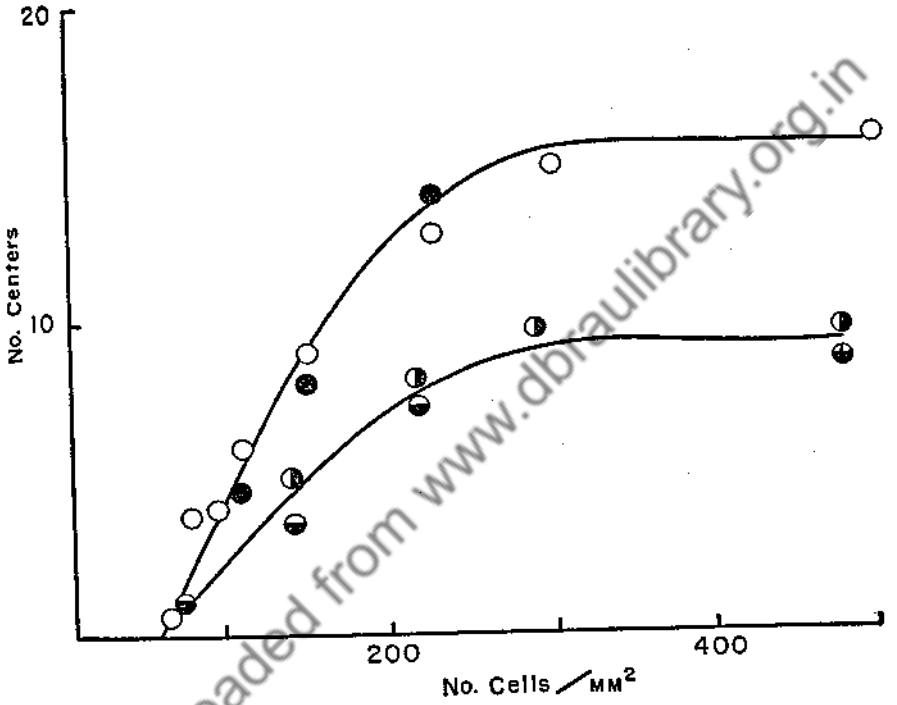


Fig. 9. Relation of the number of centers evoked by fruity initiator cells in mixtures with wild type and the population density of the mixture (Sussman, 1955). The upper curve was obtained with mixtures containing 1,000 Fty-1 cells; the lower with 500.

the mutants. Clearly, the mutant did contribute a large number of initiators to which the wild type responders could react. In order to abstract from these data the precise number of centers evoked by fruity initiators, the centers produced in the wild type control were subtracted from those of the mixture at the same density. These resultants provided points for a new curve relating the number of mutant-induced centers to the population density of the mixture. Fig. 9 shows two such curves; one for mixtures containing 1000 fruity cells and the other, 500. With increasing density, the number of centers evoked by mutant

initiators increased until a plateau was reached at about 300 cells/mm². Note that at higher densities the mutant-instigated centers remained constant in number while from Fig. 8 it is seen that those evoked by the wild type initiators declined. In other experiments this constancy upon the part of the mutants was shown to persist at densities in excess of 1100 cells/mm². Thus, the effect of high density upon center formation appears to depend in large measure upon the nature of the initiator cells.

TABLE VII. Centers evoked by fruity initiator cells in mixtures with wild type (Sussman, 1955).

Number of Fty-1 cells	Number of centers at plateau	Ratio centers : cells
1,000	15.8	1 : 63
	15.6	1 : 64
750	11.9	1 : 63
	14.6	1 : 50
500	8.8	1 : 57
	9.8	1 : 51
	Mean	1 : 58

Plateau values for the number of mutant-induced centers were found to be proportional to the number of fruity cells present in the mixture. Table VII summarizes numerical data from six determinations. The ratio of centers evoked by fruity initiators to the number of mutant cells present was 1 : 58. It is certain therefore that a large part of the mutant's capacity to produce huge numbers of centers is due to a significant increase in the number of initiators over the number present in the wild type populations. However, the fact that when fruity responders served as the test system, a ratio of 1 : 24 was obtained as compared with 1 : 60 when wild type so served indicates that part of that increase is due to superior sensitivity of the mutant responders to the aggregative stimulus.

Table VIII shows the results of experiments in which different numbers of mutants were mixed with 10,000 or 25,000 wild type and were dispensed at a density of 400 cells/mm², more than enough to attain the plateau values. The ratio of centers evoked by fruity initiators to fruity cells present was constant regardless of the absolute number of

wild type in the test system. Further, 600 fruity cells if mixed with 25,000 wild type at a density of 400 would be much more separated from one another than if mixed with only 10,000 wild type at the same density. The fact that the ratio did not depend upon the state of dispersion of the fruity cells in the mixture indicates that the mutant initiators act singly to evoke centers just as do the wild type initiators.

TABLE VIII. Centers evoked by fruity cells in mixtures with (A) 10,000 and (B) 25,000 wild type at a density of 400 cells/mm² (Sussman, 1955).

A		B	
Number of Fty-1 cells	Ratio centers : cells	Number of Fty-1 cells	Ratio centers : cells
1,200	1 : 59	1,200	1 : 61
1,100	56	1,100	59
1,000	62	1,000	60
900	65	900	63
800	61	800	60
700	64	600	58
600	58		
500	62		
400	64		
	Mean 61		Mean 60

It is of interest to note that extracts of the fruity cells, when incorporated into streptomycin-fortified washed agar have been found to augment greatly the number of centers formed by the wild type. Our best preparations have yielded increases up to eleven-fold. Viable counts of wild type on the extract agar showed no increase in the number of cells nor were any viable fruity carried over in the extract. The biological and chemical properties of this extract are under current investigation, but it is still too early to make definite statements about them.

Plate III shows photomicrographs of two representative mutant fruits. The latter contained about 50 spores, 4-6 stalk cells, and 1 or 2 basal disc cells; the first, 9 spores, 2 stalk cells, and 1 basal disc cell. A study of the comparative anatomy of these and other mutant fruits was undertaken (Sussman, 1955b). Corresponding data for the wild type were primarily obtained from the publications of Raper (1941)

and Bonner and Slifkin (1949). The results indicate that the absolute numbers of cells in the morphogenetic assembly exert no more than a very minor influence upon either the cellular morphology of the fruits or their gross proportions. The presence of rigid regulatory processes is not unknown in morphogenetic systems as is clearly evident in the embryological literature. But perhaps the slime molds represent an extreme case for the constancy of morphogenetic properties. At one end of the gamut are wild type fruits on growth plates (where the population density is enormous), containing hundreds of thousands of cells and measuring several millimeters in height. At the other end are the Fty-1 fruits on washed agar at optimal density containing 12 cells or less and measuring 40 to 60 *microns* in height—a difference of at least 10,000-fold in cell numbers and of 100-fold in size.

The fruity mutant has special pertinence for the problem of regulation. Studies by Raper (1940a) and Bonner (1944) have shown that the fate of a cell in the ultimate fruiting body is determined by its geographical position in the pseudoplasmodium, which in turn is governed by the relative order in which that cell entered the aggregate. This fate may be altered predictably by changing the position of the cell. It would appear, therefore, that the myxamoebae become spores, stalk or basal disc cells, and do so in definite proportion, by virtue of interactions between different parts of the assembly. The question arises as to whether such interactions represent mass effects of one large group of cells upon another or whether they can be limited to small groups of effectors and effectees. The fruity mutant tells us that not only can these interactions involve limited groups of cells, but in this case they must. In the fruit pictured in Plate III, 1, nine myxamoebae apparently informed two others that they were to become stalk cells or vice-versa. The interest in these proceedings springs from the inference that in such interactions the effector cells have to provide a powerful impetus or else the effectees must be exceedingly sensitive to the signal. The possibility then arises that one could conceivably use a micro-system of this kind with its attendant simplicity to dissect the complex of interactions that occur.

One project looked forward to with great anticipation on our part is the dissection of the tiny fruits so as to isolate each of the component cells in a viable condition. The growth of these isolates will yield clones with which to test whether every component cell in the fruit can provide its progeny with a complete morphogenetic potential. The transition from stalk or basal disc cell to vegetative myxamoebae should be of

cytological interest. A corresponding study has already been made with the wild type *D. mucoroides* and *D. purpurem* (Sussman, 1951). It is known that at least a good part of the stalk cells in the parent fruits give rise to normal clones, but total surveys could not be taken.

VI. DISCUSSION

Demonstrable cases of interactions during development are of course not unique to the slime molds. The embryological literature is replete with similar examples. These include relatively circumscribed effects encountered within or between developmental subsystems as, for example, the induction of lens development in ectoderm by the optic vesicle of the chick embryo (McKeehan, 1951), which it is interesting to note appears to proceed by direct contact, or the influence of the neural tube and notochord of the same organism upon the axial skeleton (Watterson et al, 1954). More generalized effects are apparent including that of the disappointing but highly instructive embryonic organizer in amphibia. In addition, many interactions have been uncovered which lead to developmental inhibitions. A case in point is the existence of dominance relationships in coelenterates that can be destroyed by erecting barriers against free physiological contact between component parts of the organism (Barth, 1938). Another is the extremely stimulating work of Yamada (1940) who established the existence of a morphogenetic hierarchy within the mesodermal complement of salamanders and who showed the ability of notochord in contact with the mesodermal isolates to raise the potential of each without disturbing the developmental peck order.

Indeed, some aspects of these interactions have already entered the kingdom of developmental theory. The role of physiological competition in determining dominance relationships and in limiting developmental potentials within morphogenetic fields has been given an inspired formal treatment (Spiegelman, 1945). Perhaps developmental experimentation is still not sophisticated enough to take advantage of this approach but its potential heuristic value is enormous. In this connection it is noteworthy that several phases of slime mold development, and in particular the pseudoplasmodial stage, fulfill the requirements of morphogenetic fields. A number of cytological and physiological markers are available with which to score the individual cells in the assemblies, and the existence of dominance relationships within the fields has been established (Raper, 1941, Bonner, 1950). The uncoupling of development from net cell growth during many of these phases and the ease of

mechanical manipulation contribute additional advantages. It would appear therefore that the slime molds can provide ideal material for the amplification of biological field theory.

A great deal of speculation has also been offered (Weiss, 1949, 1950) to account for interactions that lead not to limitations of developmental potentials but to the induction of additional heterogeny. Particular concern has been paid to direct contact phenomena. The explanations are largely based upon the assumption of stereochemical reactions at cell interfaces which in turn might lead to progressive constraints upon the structure of cell interiors. One could equally well invoke the possibility of cytoplasmic exchanges as the basis of direct contact inductions. The introduction of persistent phenotypic determinants by these means has ample precedent in microbiology (Sonneborn, 1946, 1950, 1953; Ephrussi, 1951; Spiegelman, 1951; Sager, 1954; Mitchell et al, 1952). Other possibilities exist and only decisive experimentation will reveal whether any or all of them are valid. The morphogenetically deficient mutants might serve as one of the routes to this decision.

There exists then a most considerable body of data and insight that may provide lessons to be learned in conducting future investigations into interactional phenomena. The first of these is the danger, so clearly demonstrated in the case of the embryonic organizer, of pursuing effects between intricately organized and extended developmental subsystems. Such assemblies are largely autonomous in the developmental sense and apparently require little more than a trigger to set them off. Consequently, their study can lead to a great deal of knowledge of the trigger but little of the gun. The danger would seem to be remote in the case of the slime molds. The small numbers of cells involved and the relative simplicity of the morphogenetic product lend a measure of reassurance. This is particularly true of the fruity mutant. In addition, the synergistic response patterns of the morphogenetically deficient stocks indicate a high level of specificity.

Second, most of the embryological studies of interaction thus far accomplished have been centered upon mass action phenomena. The stimuli, so far as can be judged, are supplied through the cumulative efforts of a great many cells and they affect similarly large populations usually in an already advanced state of organization. The attendant complexity has precluded quantitative study of the kinetics of the interaction and its extent in terms of the individual cells. It may be argued

that in most developmental systems the organization is not the simple sum of its parts and that the resultant possesses an integrated character destroyed by fragmentation. Yet, at its base, development involves the appearance of phenotypic divergence in *cells*. It would seem therefore that simpler systems in which a cellular approach can now be made might serve at least as very valuable supplements and perhaps give rise to fundamental insights. The slime molds may prove of value in this respect. The ability of the experimenter to separate cells at any stage of the development and to grow them in clonal isolation opens the way to the application of the methodology of microbial genetics in attempts to understand the genetic bases of the differentiation.

Also readily apparent is the need for complete physiological descriptions of interactional phenomena: the nature of the chemical agent, the conditions and biochemical pathways through which it is synthesized by the effector cells and its mode of transport *in vivo;* the receptor mechanisms of the stimulated cells and the enzymatic alterations induced or selected thereby. The direct contact interactions are the knottiest problem in this respect but also the most challenging. As mentioned, specific information about direct contact exchanges has been gained in microbiology but with little understanding as yet, excepting the brilliant investigation of *Paramecium aurelia* by Sonneborn and his collaborators.

Judged by these criteria the studies of interactional phenomena in the slime molds are still very primitive. The phenomena have barely been demonstrated and their genetic and physiological depths have not been plumbed to any degree. Their potentialities, however, remain bright.

A last point may be raised concerning the pertinence of the study of slime molds to developmental physiology and epigenetics in general. The relatively feeble morphogenetic capacity of these organisms when contrasted with the complexities of embryogenesis and regeneration might well lead one to conclude that any mechanistic analogies to be drawn between them would be tenuous at best. And yet, the prodigious predictive value of comparative biochemistry and anatomy to the biological discipline encourages the belief that a comparative approach can be equally fruitful to developmental study. Further, the evolution of stable multicellular existence remains one of the most intriguing problems in biology. The slime molds and related *Protista* provide material wherein this problem may be faced.

BIBLIOGRAPHY

Arndt, A. 1937. Untersuchungen über *Dictyostelium mucoroides*. *Arch. mikroscop. Anat. Entwicklungsmech. 136*, 681-747.

Barth, L. G. 1938. Quantitative studies of the factors governing the rate of regeneration in Tubularia. *Biol. Bull. 74*, 155-177.

Bonner, J. T. 1944. A descriptive study of the development of the slime mold *Dictyostelium discoideum*. *Amer. J. Bot. 31*, 175-182.

Bonner, J. T. 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool. 106*, 1-26.

Bonner, J. T. 1949. The demonstration of acrasin in the later stages of the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool. 110*, 259-272.

Bonner, J. T. 1950. Polarity in the slime mold *D. discoideum*. *Biol. Bull. 99*, 143-151.

Bonner, J. T. 1951. The pattern of differentiation in ameboid slime molds. *Am. Nat. 86*, 79-89.

Bonner, J. T., and M. K. Slifkin. 1949. A study of the control of differentiation: the proportions of stalk and spore cells in the slime mold, *D. discoideum*. *Am. J. Bot. 36*, 727-734.

Bradley, S. G., and M. Sussman. 1952. Growth of ameboid slime molds in one-membered cultures. *Arch. Biochem. Biophys. 39*, 462-463.

Bradley, S. G., and M. Sussman. 1955. Environmental factors affecting the aggregation of the cellular slime mold, *D. discoideum*. In manuscript.

Braun, W., R. Goodlow, M. Kraft, R. Altenbern, and D. Mead. 1951. Effects of metabolites upon interactions between variants in mixed *Brucella abortus* populations. *J. Bacteriol. 62*, 45-52.

Ephrussi, B., and H. Hottinguer. 1951. On an unstable cell state in yeast *Cold Spring Harbor Symp. Quant. Biol. 16*, 75-87.

McKeehan, M. S. 1951. Cytological aspects of embryonic lens induction in the chick. *J. Exp. Zool. 117*, 31-64.

Mitchell, M. B., and H. K. Mitchell. 1952. A case of material inheritance in *Neurospora crassa*. *Proc. Nat. Acad. Sci. 38*, 442-449.

Olive, E. W. 1902. A monograph of the Acrasiae. *Proc. Bost. Soc. Nat. Hist. 30*, 451-513.

Raper, K. B. 1937. Growth and development of *D. discoideum* with different bacterial associates. *J. Agric. Res. 55*, 289-316.

Raper, K. B. 1939. Influence of culture conditions upon the growth and development of *D. discoideum*. *J. Agric. Res. 58*, 157-198.

Raper, K. B. 1940a. Pseudoplasmodium formation and organization in *D. discoideum*. *J. Elisha Mitchell Sci. Soc. 56*, 241-282.

Raper, K. B. 1940b. The communal nature of the fruiting process in the Acrasiae. *Amer. J. Bot. 27*, 436-448.

Raper, K. B. 1941. Developmental patterns in simple slime molds. Third Growth Symposium. *Growth 5*, 41-76.

Raper, K. B., and C. Thom. 1941. Interspecific mixtures in the Dictyosteliaceae. *Am. J. Bot. 28,* 69-78.

Raper, K. B., and D. Fennell. 1952. Stalk formation in *Dictyostelium. Bull. Torrey Bot. Club 79,* 25-51.

Sager, R. 1954. Mendelian and non-Mendelian inheritance of streptomycin resistance in *Chlamydomonas reinhardi. Proc. Nat. Acad. Sci. 40,* 356-363.

Shaffer, B. M. 1953. Aggregation in cellular slime molds: *in vitro* isolation of acrasin. *Nature 171,* 975.

Singh, B. N. 1947a. Studies on soil Acrasiae. *J. Gen. Microbiol. 1,* 11-21.

Singh, B. N. 1947b. Studies on soil Acrasiae. II. *J. Gen. Microbiol. 1,* 361-367.

Sonneborn, T. M. 1946. Experimental control of the concentration of cytoplasmic genetic factors in Paramecium. *Cold Spring Harb. Symp. Quant. Biol. 11,* 236-255.

Sonneborn, T. M. 1950. Cellular transformations. *Harvey Lect. 44,* 145-164.

Sonneborn, T. M. 1953. Change in ciliary serotype following cytoplasmic exchange in *P. aurelia. Microb. Gen. Bull. 7,* 22.

Spiegelman, S. 1945. Physiological competition as a regulatory mechanism in morphogenesis. *Quart. Rev. Biol. 20,* 121-146.

Spiegelman, S. 1951. The particulate transmission of enzyme forming capacity in yeast. *Cold Spring Harb. Symp. Quant. Biol. 16,* 87-98.

Spiegelman, S., R. R. Sussman, and E. Pinska. 1950. On the cytoplasmic nature of long term adaptation in yeast. *Proc. Nat. Acad. Sci. 36,* 591-606.

Sussman, M. 1951. The origin of cellular heterogeneity in the slime molds Dictyosteliaceae. *J. Exp. Zool. 118,* 407-418.

Sussman, M. 1952. An analysis of the aggregation stage in the development of the slime molds Dictyosteliaceae. II. Aggregation by mixtures of *D. discoideum* wild type and Aggregateless mutants. *Biol. Bull. 103,* 446-457.

Sussman, M. 1954. Synergistic and antagonistic interactions between morphogenetically deficient variants of the slime mold *D. discoideum. J. Gen. Microbiol. 10,* 110-121.

Sussman, M. 1955a. Developmental physiology of the ameboid slime molds. In *Biochemistry and Physiology of Protozoa,* vol. II, edited by S. H. Hutner and A. Lwoff. Academic Press, New York.

Sussman, M. 1955b. Fruity and other mutants of the cellular slime mold *D. discoideum:* a study of developmental aberrations. *J. Gen. Microbiol. 13,* 295-309.

Sussman, M., and E. Noel. 1952. An analysis of the aggregation stage in the development of the slime molds Dictyosteliaceae. I. Populational distribution of the capacity to initiate aggregation. *Biol. Bull. 103,* 259-268.

Sussman, R. R., and M. Sussman. 1953. Cellular differentiation in the Dictyosteliaceae: heritable modifications of the developmental pattern. *Ann. N.Y. Acad. Sci. 56, No. 5,* 946-960.

Sussman, M., and S. G. Bradley. 1954. A protein growth factor of bacterial

origin required by the cellular slime molds. *Arch. Biochem. Biophys. 51*, 428-435.

Sussman, M., and F. Lee. 1955. Interactions among variant and wild type strains of cellular slime molds across thin agar membranes. *Proc. Nat. Acad. Sci. 41*, 70-78.

Watterson, R. L., J. Fowler, and B. J. Fowler. 1954. The role of neural tube and notochord in development of the axial skeleton of the chick. *Am. J. Anat. 95*, 337-400.

Weiss, P. 1949. Growth and differentiation on the cellular and molecular levels. *Exp. Cell. Res., Suppl. 1*, 475-482.

Weiss, P. 1950. Perspectives in the field of morphogenesis. *Quart. Rev. Biol. 25*, 177-198.

Yamada, T. 1940. Beeinflussung der Differenzierungsleistung des isolierten Mesoderms von Molchkeimen durch zugefügtes Chorda- und Neural-material. *Fol. Anat. Jap. 19*, 131-198.

VII. NEW APPROACHES TO THE PROBLEM OF EMBRYONIC INDUCTION

BY M. C. NIU [1]

After the discovery of the organizer in urodele development (Spemann and Mangold, 1924), embryonic induction has become one of the most studied aspects of experimental embryology. During the past twenty years embryologists throughout the world have contributed a large amount of new information to the subject, much of which has fortunately been covered in careful reviews (Holtfreter, 1951; Holtfreter and Hamburger, 1955). Results reported in the last few years have also been brought together by Needham (1955). According to these reviews, our information of embryonic induction, particularly its chemical aspects, appears diffuse and conclusions can hardly be drawn. It seems that further progress on the subject, as pointed out by Holtfreter, may be made only with the development of new analytical technique.

Since this paper concerns new approaches, an explanation of the conventional methods employed in the study of embryonic induction becomes necessary. Originally the experiments were performed by grafting the organizer tissues to new sites in host embryos and by implanting homoplastically and xenoplastically into the blastocoel. Several years later, a new technique was introduced (Holtfreter, 1933) which involves wrapping young ectoderm around a piece of organizer, living or dead, and culturing it in standard Holtfreter solution. This technique has since been modified somewhat by "sandwiching" a piece of inductor tissue between two sheets of ectoderm. For testing inductive activity of a known substance, the compound embedded in agar or albumen (Needham, 1942), or a pellet of the compound (Yamada and Takata, 1955), was handled in the same way as inductor tissue.

These experimental approaches were based upon the hypothesis that embryonic inductor can act only when in direct physical contact with

[1] Department of Biological Sciences, Stanford University. This investigation was supported in part by grants from the Committee on Growth of the National Research Council, and the Rockefeller Foundation. The author takes pleasure in acknowledging his indebtedness to Professor V. C. Twitty for his sustained interest and enthusiastic support in the work reported here. The latter part of the results were compiled during tenure of a Guggenheim Fellowship at the Rockefeller Institute for Medical Research.

the reacting tissue (e.g. Holtfreter and Hamburger, 1955). Separation of the two tissues by the smallest gap or the interposition of the thinnest cellular or artificial barriers has invariably blocked induction (Brachet, 1950; Weiss, 1950; McKeehan, 1951; Grobstein,[2] 1955). So consistent have been these findings that, in spite of the large amount of work on the chemistry of inductors, it has not been demonstrated conclusively that diffusible substances (e.g. Tung et al., 1949) are involved in induction. The work of Barth (1941) and Holtfreter (1944) on neuralization of young ectoderm *in vitro* has shown that induction may occur in the absence of an externally applied inductor. According to Holtfreter (1945 and 1947), however, the induction is probably indirect. Toxic changes from variation of pH, for instance, produce some cytolysis, or sublethal cytolysis, and it is the product of this cytolysis which in turn causes adjacent cells to differentiate. As long as the induction is produced by indirect means, the process may actually not be related to what is going on during normal development.

The first phase of a renewed attack on this problem was the study of differentiation of isolated ectodermal cells. In capillary tubes filled with coelomic fluid drawn from spawning female newts, isolated ectodermal cells became in a few cases spindle-shaped, presumably myoblasts. Thus, it appeared that naturally occurring substances in coelomic fluid were evoking a response in ectodermal differentiation. Furthermore, isolated single tumor cells were found to grow only in a medium drawn from established cultures (Sanford, Earle, and Likely, 1948). From these findings, it was reasoned that organizer tissue, grown *in vitro,* might also condition the culture medium. It seemed conceivable that young ectodermal cells might actually be induced to differentiate in the medium withdrawn from organizer cultures. This relationship might thus be formulated as follows:

inductor substance in solution + totipotential ectoderm → differentiation

When presumptive ectoderm remains constant (i.e. using young ectoderm of the same age), the type of differentiation is bound to depend on the nature of the inductors. Specific inductor would produce specific differentiation. Accordingly the type of differentiation depends upon

[2] It is true that induction occurs *in vitro* in mammalian tissues with a filter lying between the inducing and reacting tissues; however, the cytoplasmic processes within the pores of the filter offer the possibility that contact may again be involved. In fact, Grobstein (unpublished) has advanced the idea that induction is mediated through the matrix of the ground substances produced by tissue cells. Accordingly, contact in a modified form may indeed be involved in this instance.

the nature of the inductor. This approach may lead us to the achievement of directed differentiation.

The second phase in formulation of a new approach concerned the choice of material. Since Holtfreter had found that the cells of *Triturus torosus* ectoderm, in contrast to those of *Amblystoma punctatum,* do not cytolyse *in vitro, torosus* seemed promising. It is, however, essential to eliminate the surface coat of the ectoderm which is known to be nonpenetrable by chemical substances (Holtfreter, 1943). The most effective method is to cut the thick layer of young ectoderm into very tiny pieces. When they round up, the surface coat is insufficient to cover even half the outer surface of such ectodermal explants, thus permitting any agent in the medium to act directly upon exposed cells. As a matter of fact, the remnant of the surface coat actually tends to turn inward into the center of the explant rather than to stay outside (Plate I, 1). In brief, the adopted procedure is to culture, in hanging drops at 19°C, large pieces of inductor tissue together with tiny pieces of the presumptive ectoderm that can readily be excised by micro-surgical techniques. It must be emphasized that throughout these experiments the two tissues were situated separately and had no physical contact with one another.

The medium used for this study is modified Holtfreter solution developed by us some eight years ago for the cultivation of the neural fold (published by Flickinger, 1949; Niu and Twitty, 1953). The distinguishing features of this modified solution are: (1) the magnesium ions promote significantly the attachment and spreading on a glass surface of young gastrula ectoderm (unpublished, Niu and Twitty), and (2) the phosphate buffer stabilizes the pH of the medium at 7.8 ± 0.15.

Early in our study, both inductor and reacting tissues (presumptive ectoderm of stage 10 — beginning gastrula) were taken from *torosus* embryos, the inductor being confined to the dorsal half of Spemann's organizer area (dorsal lip of the blastopore, stages 10–12). Later, the source of inductor tissue was extended progressively to the trunk medullary plate including some of the adjacent neural folds (stages 15–17), the posterior portion of the medullary plate (potential mesoderm, stages 15–16), notochord (stages 16–18), combinations of all of these, and finally endoderm (stages 16–19). In addition to *T. torosus, T. rivularis,* axolotl (*Amblystoma mexicanum*), and *A. tigrinum* were used. It should be mentioned that the role of endoderm and neural fold as inductors is being studied by Miss L. A. Forkgen.

I. INDUCTION IN CONDITIONED MEDIUM

The rate and pattern of pigment-cell development in neural fold (crest) cultures recapitulate those shown *in vivo* (Twitty, 1945). Similarly, the differentiation of the developing inductor tissue in the modified salt solution is found to keep pace with *in vivo* development. This equivalence of *in vivo* and *in vitro* development lasts 2 to 4 weeks according to the type of the inductor tissue. During this time, the inductor explant differentiates according to its source into various tissues and cells. Dorsal lip gives rise to myoblasts, notochordal mass, mesenchyme, and sometimes to nerve tissue and pigment cells; medullary plate and fold to neural tissue and an outgrowth of chromatophores; posterior portion of the medullary plate (PMP) to myoblasts, and also frequently mesenchyme, chromatophores, notochordal mass, or even some neural tissue; notochord to rod-shaped or massive chordal structures; neural fold to neural tissue and an outgrowth of chromatophores; somitic blocks to myoblasts and sometimes mesenchyme; and endoderm to thin endodermal sheets.

When large pieces of inductor and tiny pieces of young ectoderm are explanted simultaneously into hanging drops of modified salt solution (referred to later on as 0 day cultures), both explants attach to the glass and develop during the first few days. The inductor explant continues to grow, and to differentiate according to its source. After initially spreading into thin sheets, at least 95% of the ectodermal explants soon retract into spheres and usually become detached. Cilia develop and thus the ectodermal explants become free swimming vesicles which eventually burst. There are rare instances, however, in which the ectodermal explants behave similarly to those in the next experiment to be described and undergo differentiation.

When tiny ectodermal pieces are isolated by themselves in the modified salt solution, there is a greater tendency toward cell dissociation with a small number of loose cells appearing at the margin of the explant. These cells frequently become detached and soon cytolyse, releasing their contents into the medium. During the past three years, more than 2,000 such controls were made, and in not more than a half-dozen cases was there the slightest indication of differentiation. Apparently the products of these few cytolysed cells are insufficient to induce the neighboring cells to differentiate.

If the failure of induction in 0 day cultures is due to insufficient conditioning of the medium during the short period when the ectoderm is

still competent to react, it should be possible to obtain induction in cultures where the developing inductor explant has been developing over a longer period. Young ectodermal explants introduced into 7-10 day old cultures of inductor tissues, were found to undergo differentiation (Niu, 1953; Niu and Twitty, 1953, and unpublished) in the complete absence of physical contact between the two explants. The percentage of induction occurring varied considerably according to (1) the inductor tissue and (2) the species of young ectoderm employed. The best series on record shows that ectodermal differentiation in cultures is as high as 90%.

In the most representative cases, the behavior of the ectodermal explant is remarkably similar to that of an explant of the neural fold. The ectodermal explant becomes intimately attached to the cover slip and spreads as does the control in the unconditioned salt solution. Three to six days later, amoeboid cells begin to emigrate and gradually a good sized outgrowth is established. Practically all of these cells differentiate into highly branched pigment cells. Nerve fibers radiate out of the central residual mass (Plate II, 1). Variations of this pattern are: (1) the entire outgrowth of the ectodermal explant differentiates into chromatophores only (Plate II, 2); (2) the explant may remain compact and give rise to radiating nerve fibers only (Plate III, 2); (3) occasionally myoblasts develop, and (4) rarely the cells of the whole ectodermal explant become widely scattered and, in a few days, the majority of them round up and soon detach. The few that remain attached become pigmented. However, on two occasions several scattered cells reaggregated into a tiny cluster from which a few nerve fibers emerged. Regardless of the source of the inductor explant, the young ectoderm has shown the same pattern of behavior.

It seems clear that the ectodermal differentiation is attributable to a conditioning of the medium by the inductor tissue, and not to a nonspecific, toxic effect of the modified salt solution. The explants appear to be completely healthy and cell dissociation or other signs of injury are definitely rarer than in unconditioned medium in which ectodermal differentiation does not occur.

It is assumed then that the developing inductor "conditions" the medium by releasing inductor substances into it. These substances accumulate until a threshold concentration sufficient to exert an influence on the ectodermal cells is reached. The ectodermal cells, otherwise capable of forming only a simple epithelium *in vitro*, then differentiate into a variety of cell types. The intrinsic factors responsible for ectodermal

differentiation into simple epithelium are therefore overridden, or inhibited, and replaced by extrinsic ones which direct the ectodermal cells into new channels of differentiation. If true, this would imply that the inductor substances initiate a series of processes leading to specific histological differentiation.

Although the agents responsible for induction *in vitro* appear to be diffusible substances in the medium, the possibility that the actual physical presence of the developing inductor is essential remains to be excluded. To eliminate this possibility, the medium in which the inductor explant had been growing for 10 days was withdrawn and used to culture new ectodermal isolates. These differentiated as if explanted into established cultures of developing inductor.

Let us examine the cell population involved in these experiments. As shown in Plate I, 1, the tiny explant contains no more than 15-20 cells. When larger, the explants tend to be surrounded by surface coat, and consequently fail to undergo differentiation. When induction occurs in these small explants the outgrowth may contain more than one hundred pigment cells (see Plate II). The increase in number shows that cell division is active prior to the onset of differentiation. Plate I, 2, shows in cross section some ectodermal explants that have been developing in conditioned medium for about three weeks. Comparing with Fig. 1 on the same plate, one notices immediately the large increase in number and the small size of the constituent cells. Particular attention is called to the figure on the left showing that the ectodermal explant is a vesicle composed of a large number of small cells. Similarly, those ectodermal vesicles in the controls of the modified salt solution are made of small epithelial cells. It appears that, irrespective of the medium, cells of the ectodermal explants undergo active division, but the explants in the controls do not differentiate. To demonstrate this point more crucially, single ectodermal cells were explanted into capillary tubes filled with conditioned medium. Again division was frequent (Plate III, 1) and these cells appear to develop into propigment cells. Accordingly, the inductor substances of the conditioned medium are indeed differentiators and not growth promoters.

In order to reveal how much the age, or degree of differentiation, of the inductor explant may affect the induction, older cultures (12-16 days) of posterior medullary plate (PMP) were employed (Table I). PMP was chosen because it frequently gives rise to an outgrowth of myoblasts and also because it is a very active inductor *in vivo*.

TABLE I. Induction of myoblasts in older cultures of posterior medullary plate (PMP).

Series of inductor	Total	Induction * Myoblasts (% of total)
No. 35: *torosus* PMP (12 days)	70%	35·7
No. 47: *rivularis* PMP (15 days)	70%	38.5
No. 48: *torosus* PMP (12 days)	66%	73

*Based on 60 cultures per series.

In contrast to the rare occurrence (not more than 5%) of induced myoblasts in the 7-10 day old cultures mentioned previously, myoblast differentiation now increases to as much as 73% of the total inductions obtained (Plate IV, 1 and 2). This difference is caused apparently by a difference in age of the inducing culture. The possible ways by which the inductor substances of the culture medium can produce such differences in ectodermal differentiation are (1) difference in concentration of a single substance and (2) the existence of two or more different substances. For reasons that will be given later (in connection with homeogenetic induction and spectrophotometric measurements), it is believed that the difference is not quantitative in nature.

Recognizable cell-types were usually seen in the outgrowth of the developing PMP explants about 10 days after explantation. It appears that before the onset of differentiation there is a period in which cell division is active, particularly during the period when cells are emigrating from the explant. It follows, then, that the inductor substances in the 10-day-old cultures of PMP are being released by cells in the growth phase, while in 12-16 days cultures they are being released by cells actively engaged in differentiation. Since actively growing tissue is known to be rich in nucleic acids, and cells in the process of differentiation are definitely active in specific protein synthesis, it is quite likely that different substances are being liberated by the young and the older PMP cultures respectively. It is interesting to note that mybolast differentiation is seldom induced in young ectoderm by older cultures (12-16 days) of medullary plate and fold. Instead, neural tissue and pigment cells are usually induced. Since the medium conditioned by developing PMP tends to induce young ectoderm to differentiate into muscle cells, we are apparently dealing with an example of homeogenetic induction *in vitro*.

The demonstration of homeogenetic induction *in vitro* is significant. In the first place it suggests to us a simple method by which young ectoderm may be induced to differentiate in either of two directions. Secondly, homeogenetic induction implies the presence of specific inductor substances. These specific substances which are released by cells engaged in specific cytoplasmic synthesis induce the ectoderm to differentiate into corresponding cell-types. It appears that the inductor substances in the medium of older PMP cultures differ from those of older cultures of medullary plate and fold. The difference may be small, and therefore not easily detectable biochemically. In passing it may be mentioned that in experiments with fixed adult tissues and with various preparations of nucleoproteins and nucleic acids, we have frequently obtained ectodermal differentiation into neural tissues and pigment cells, but we have failed so far to observe the appearance of myoblasts.

That both inductor and reacting tissues play a role in the frequency of embryonic induction as indicated earlier requires further explanation. The importance of these factors, together with the time relationship involved in the induction phenomenon, will be dealt with separately in the following sections.

A. The potency of various inductor cultures. Inductor cultures used in this study were made from dorsal lip, posterior portion of the medullary plate, medullary plate and fold, neural fold alone, notochord, somitic blocks, and endoderm. For the most reliable comparison, ectodermal pieces should be taken from one embryo. In view of the large number of cultures involved in this analysis, not only is this impractical, but it is even impossible to employ ectoderm from embryos of the same egg-cluster. Under these circumstances, it seems preferable to express the inductive potency in relative rather than absolute terms. A certain amount of overlapping cannot be avoided. This relative potency can be expressed tentatively as follows:

dorsal lip $\geqq$ posterior portion of medullary plate $>$ notochord $\geqq$ medullary plate and fold $>$ neural fold $>$ somites $>$ endoderm

It should be noted in passing that the more potent inductors, e.g. dorsal lip or posterior medullary plate, give rise to a greater variety of tissues and cells than do the less potent, e.g. endoderm.

B. Reactivity of young ectoderm from different species. Ectoderm from the same embryos of both *torosus* and *rivularis* were each explanted into 7–10-day-old PMP cultures of *torosus* and *rivularis* previously made under identical conditions. It was found that *rivularis*

ectoderm differentiates in a slightly higher percentage of cases than does *torosus*. Similar experiments were performed with axolotl, *tigrinum*, and *torosus* ectoderm in *torosus* PMP cultures. The differentiation of both axolotl and *tigrinum* ectoderm is higher than that of *torosus*.

C. Time required for induction. Ectodermal explants were isolated in cell-free, conditioned medium. To determine the minimum duration of exposure necessary to obtain induction, the conditioned medium in the ectoderm cultures was replaced by fresh salt solution at selected intervals. Precautions were observed to avoid any possible damage to the delicate explant during the substitution. It was found that 24 hours' exposure to conditioned medium was sufficient for *rivularis* ectodermal differentiation.

II. IDENTIFICATION OF THE INDUCTOR SUBSTANCES

As indicated at the beginning of the paper, the present confusion concerning the chemical aspect of induction is perhaps due to the lack of a simple direct technique. With the development of a new approach, we have begun a series of analytical experiments on the nature of the inductor substances present in the medium withdrawn from the established organizer cultures. The following account is surely not more than preliminary, but perhaps it indicates that the chemistry of induction can be approached by our technique with success. Needless to say, much work lies ahead of us.

For microchemical analysis, at least a few ml of the conditioned medium are required. To collect this amount from individual hanging drop cultures having a volume of 10 μl would require a tremendous amount of work. Besides, it is probable that the active substances are present in such minute quantity that the medium needs to be concentrated before any analysis could be performed. Fortunately, 20-30 pieces of organizer tissues were found to grow luxuriantly in the well of a depression slide which has a fluid capacity of 250-300 μl. Twenty such slides prepared at one time will yield, after 7-10 days, 5-6 ml of the conditioned medium, which is enough for analysis (Niu, 1955). The advantage of this method is that after withdrawal fresh salt solution may be added to the culture and 7-10 days later the medium may be withdrawn again. This process is repeated 4-6 times. The medium first withdrawn is designated as "first order" conditioned medium, the second, "second order" conditioned medium, and so forth. Routinely, the conditioned medium after withdrawal is centrifuged at low tempera-

ture (2°C) to remove any cellular debris resulting from damage produced during withdrawal. On account of the number of the organizer explants in the deep-well cultures, the occurrence of some cytolysis is probably more frequent than in the hanging drop cultures. There is, however, no ground to believe that the medium of the various orders is not equivalent to that withdrawn from the hanging drop cultures. In passing it should be mentioned that there are qualitative differences in the products of ectodermal differentiation in the successive orders of conditioned medium, but a systematic study of these differences has only begun.

The centrifuged medium is subjected subsequently to the following analyses:

A. pH determination. After each collection, the pH of the medium was measured immediately. It was found that the pH remains the same as that of the modified salt solution, i.e., 7.8 ± 0.15. Consequently, the ectodermal differentiation is not caused by a change of pH, but actually by substances released into the medium by the developing organizer explant.

B. Spectrophotometric measurements. The tissues used for cultivation in deep-wells were (1) *tigrinum* trunk and tail organizers, medullary plate and fold, and (2) *torosus* PMP, and medullary plate and fold. The medium withdrawn from these cultures was examined with the Beckman spectrophotometer. In addition, there were measurements on the first order medium collected from notochord, somite, and endoderm cultures. The general pattern of the curves obtained after plotting these data is represented by Figs. 1 and 2. From these curves it appears that the minimal absorptions of the media from first to fifth order are around 240 or 245 mμ, with the maximal at 258 or 265 mμ. Apparently they resemble those given by nucleoproteins (Caspersson, 1950; and Markham et al., 1948). Without exception, maximal absorptions of the first order in each group studied are around 258 mμ, while those of the second are around 265 mμ. This difference is significant and it may represent a difference in inductor substances present in the two media. Furthermore, these two media are important, when one considers the age factor. Medium of the second order was withdrawn from cultures about 20 days old. During these 20 days, the inductor explant grows and differentiates much as it does *in vivo*. Energy is derived from the intracellular food reserves, yolk platelets. Yolk tends to be exhausted by the end of the third or the beginning of the fourth week. After exhaustion of yolk the developing inductor explant in the medium

devoid of nutrients might be expected no longer to behave as *in vivo*. The substances released then theoretically may be different from those released *in vivo*. Consequently it seems probable that only conditioned medium of first, second, and possibly the third order would contain inductor substances present in normal development.

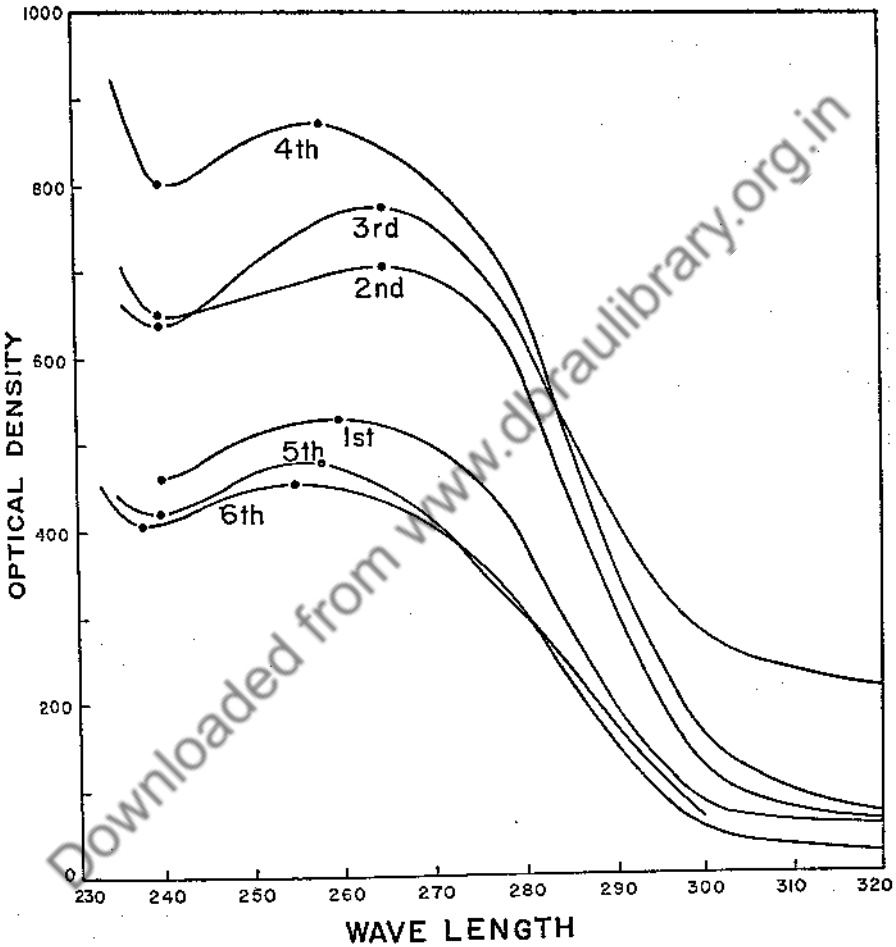


Fig. 1. UV absorption spectra of the conditioned media collected from deep-well cultures of *tigrinum* dorsal lip (55-263). 1st to 6th, the conditioned media of the first through the sixth order.

The differences in inductor substances in the medium of first and second order cultures may well reflect the differences between the young (7-10 days) and the older cultures (12-16 days) of PMP already discussed (see pages 160–161). This reflection is further shown by spectro-

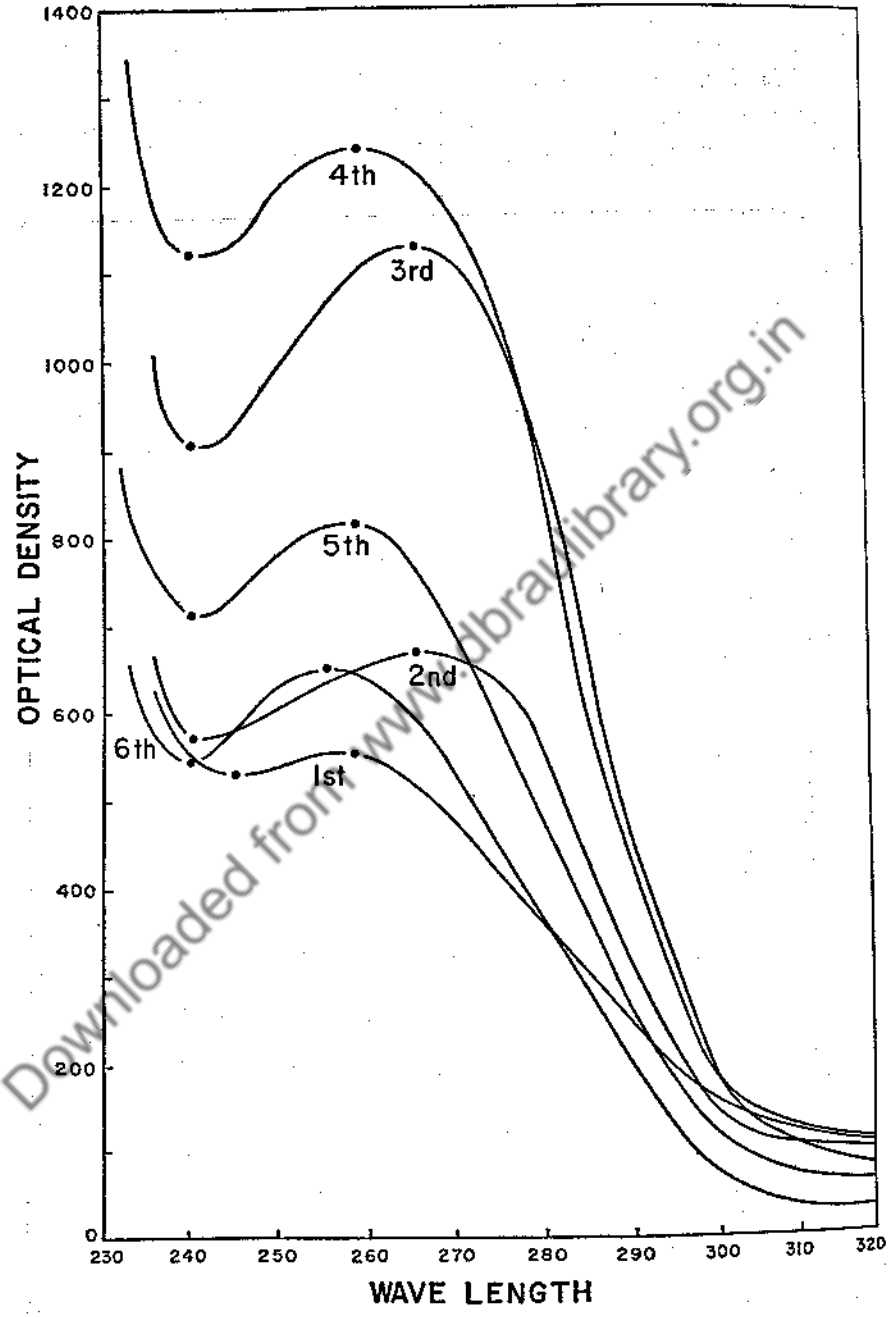


Fig. 2. UV absorption spectra of the conditioned media collected from deep-well cultures of *torosus* PMP (55-260). 1st to 6th, the conditioned media of the first through the sixth order.

photometric reading of the medium collected from the young (maximal absorptions around 258 mμ) and the older cultures (265 mμ). This would seem, therefore, consistent with the view that the medium of the first order contains a neural inductor while that of the second may perhaps contain a homeogenetic inductor substance.

As long as the chemical constitution of the different inductor substances is unknown, it could be argued that the induction of neural tissue (including pigment) and of the muscle cells may result from a difference in concentration of a single substance rather than the presence of different substances. However, in view of the concentration of substances in the young (25 μg/ml) and in the older cultures (29 μg/ml) of PMP, as given by the spectrophotometric measurements mentioned above, it seems unlikely that differentiation of neural tissue and pigment cells on the one hand, and of muscle cells on the other, can be ascribed to such a slight variation in concentration. It should be mentioned that the concentration of the inductor substances is calculated from the maximal absorption at 258 mμ and 265 mμ respectively.

Now the problem is to find whether there actually is nucleoprotein in the conditioned medium as was suggested by spectrophotometric examination. For this, we did the following experiments.

C. Dialysis. The conditioned medium collected at different times from dorsal lip and from medullary plate and fold cultures was dialysed against salt solution. The latter was changed four times in three days. Sterile precautions were observed throughout the process. After dialysis, the inductive activity of the medium was tested and differentiation was found to occur in three of the four series. This demonstrates that the inductor substances are nondialyzable, macromolecular, and, therefore, that they cannot be compounds of small molecular weight with maximal absorptions at 260 mμ, but are more probably nucleoproteins.

D. Alcohol precipitation. Based upon the maximal absorptions of each medium and assuming that the substance is pure, it was calculated that the concentration of substance varies between 0.02 and 0.047 mg/ml. The medium was centrifuged in a Spinco ultracentrifuge, Model E, at 59,600 RPM for 2 hours, but no sediments were obtained. To precipitate active substances, 7 ml conditioned medium collected at various times was first reduced to 1 ml by evaporation. Subsequently 3 volumes of ethanol (95%) were added. The conditioned medium and ethanol mixture was stored in a refrigerator over night, and then centrifuged. The alcohol in the supernatant was removed by aeration and the sediment redissolved in salt solution. The UV absorption spectrum of the

redissolved material was similar to that given by the solution from which it had been obtained. When tested with young ectodermal explants, differentiation occurred in the material that had been precipitated but not in the supernate. Thus the substances are precipitable and macromolecular, which are properties of nucleoproteins.

E. Color tests. Conditioned medium was tested with different color reagents for the demonstration of nucleic acids and proteins. The results were negative until tests were performed on concentrated samples of the medium. The concentrated medium produced a very faint light blue with the Dische test, indicating the presence of a slight amount of desoxyribonucleic acid (DNA) and dark greenish brown with the Bial test, indicating the presence of ribonucleic acid (RNA). The biuret test was positive, indicating the presence of proteins. From the readings of color intensity obtained in the Dische and Bial tests, it was calculated that the ratio of RNA to DNA is approximately 6. Since the inductive substances are discharged from cytoplasm, and DNA is usually not present in the cytoplasm of somatic cells, the presence of DNA in the conditioned medium seems to suggest that some cytolysis had occurred in the deep-well cultures.

The results of the above three experiments provide evidence that nucleoprotein is present in the conditioned medium. In an attempt to supply further evidence that the nucleoprotein in the conditioned medium is effective in induction, the effects of enzymes were investigated.

F. Enzyme studies, using ribonuclease, desoxyribonuclease, trypsin, and chymotrypsin. In experiments with *torosus* ectoderm (20 series) addition of ribonuclease to the conditioned medium greatly reduced, but did not completely prevent, induction; desoxyribonuclease slightly reduced induction; and chymotrypsin had practically no effect. When the two nucleases were added together no induction was obtained. Similar results were obtained with axolotl ectoderm (6 series). A conclusion that might be drawn from these experiments is that induction in *torosus* and axolotl by the conditioned medium is due mainly to ribonucleic acid, somewhat to desoxyribonucleic acid, but not to protein.

Although most of the experiments were done with *torosus* and axolotl, three series were performed with *rivularis*. In experiments with *rivularis* the same medium and enzymes were used, but different results were obtained: when both nucleases were added induction was diminished but not abolished, and, on the other hand, the addition of either trypsin or chymotrypsin completely prevented induction. In this species it would, therefore, appear that protein plays a part in induction. If con-

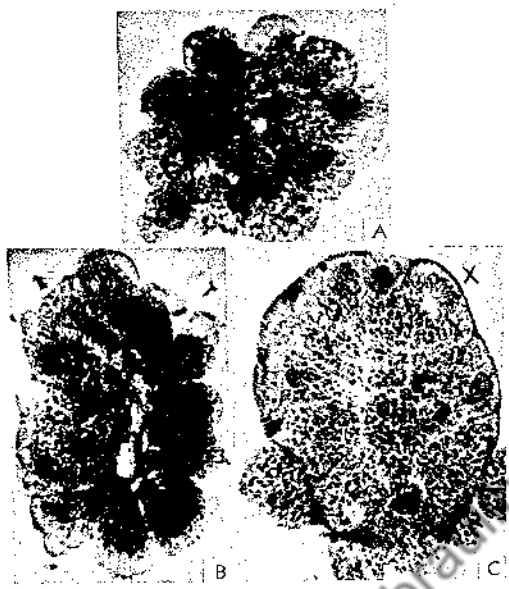


PLATE I. Fig. 1. Cross section (8μ in thickness) of 3 tiny picces of young ectoderm, 30 minutes after cutting in salt solution. A. Axolotl. B and C. *Triturus torosus*. X. Mitotic division.

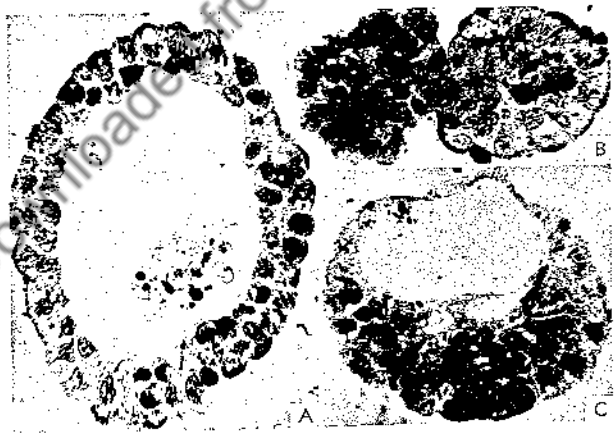


PLATE I. Fig. 2. Cross section (8μ in thickness) of 3 *torosus* ectoderm explants after 20-day cultivation in conditioned medium. A. Ectodermal vesicle. B. Explant consisting of neural and ectodermal lobes. C. Explant consisting of a naked hind-brain-like structure.

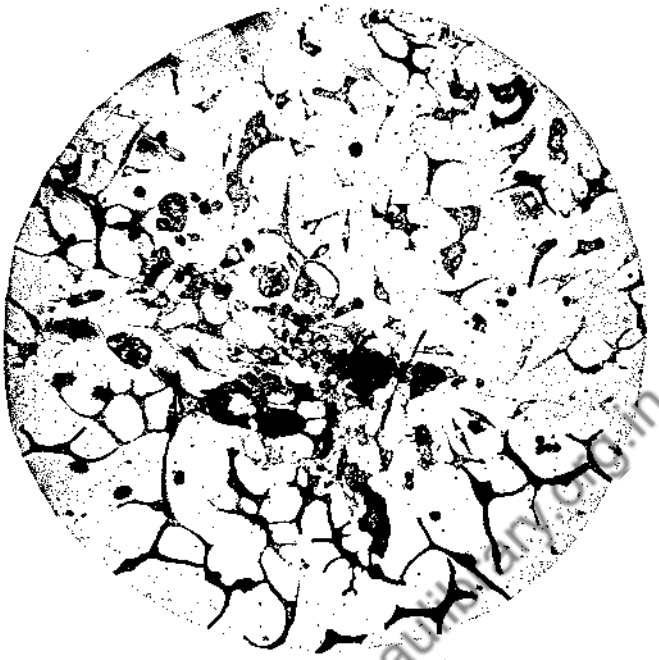


PLATE II. Fig. 1. Typical culture developed from a tiny piece of young *torosus* ectoderm after introduction into a 15-day-old culture of *torosus* trunk medullary plate and fold. Photographed 26 days after introduction of the ectodermal explant.

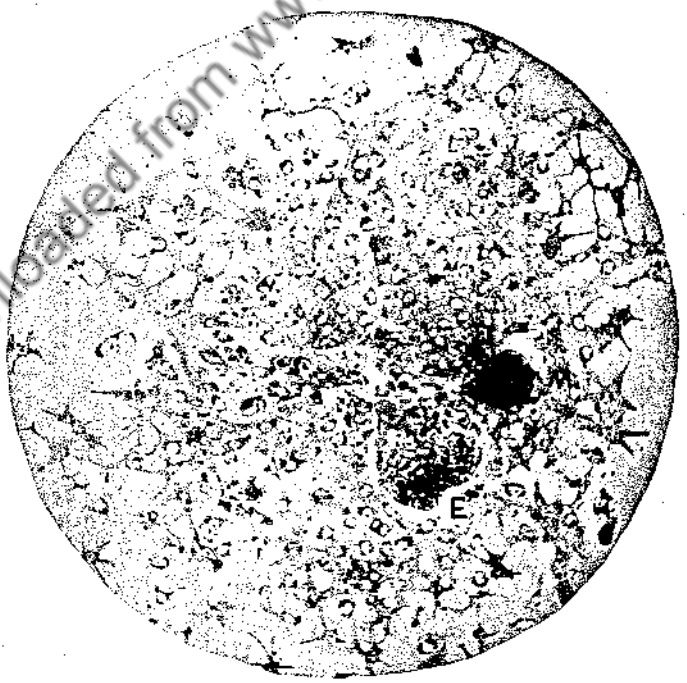


PLATE II. Fig. 2. An outgrowth of chromatophores developed from young *axolotl* ectoderm in an 11-day culture of *tigrinum* notochord. The central residual mass (M) is accompanied by a retracted epithelial sheet (E). Photographed 10 days after introduction of the ectodermal explants.

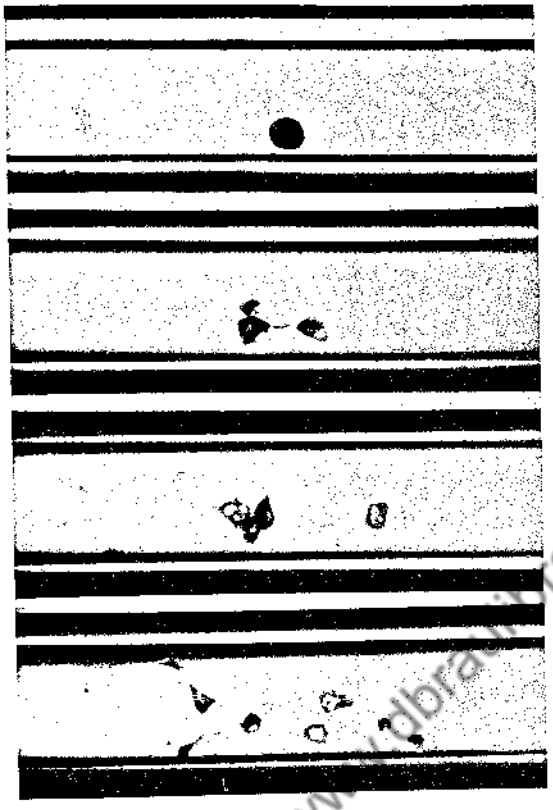


PLATE III. Fig. 1. Top to bottom: single ectodermal cell isolated into capillary tube filled with conditioned medium. Photographed at 2nd, 4th, 5th, and 7th day after explantation.

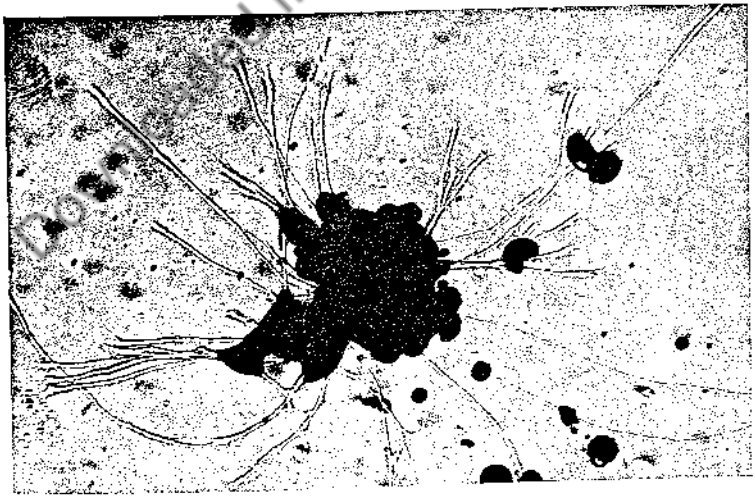


PLATE III. Fig. 2. Neural tissue with radiating nerve fibers formed from a piece of young *rivularis* ectoderm in cell-free conditioned medium. Photographed 11 days after explantation.

PLATE IV. Fig. 1. Left, explant of *torosus* PMP with outgrowth of differentiating myoblasts. Right, myoblasts developed from a tiny piece of *torosus* ectoderm introduced into the same drop 12 days after the isolation of the PMP. Photographed 23 days after introduction of the ectodermal isolate.

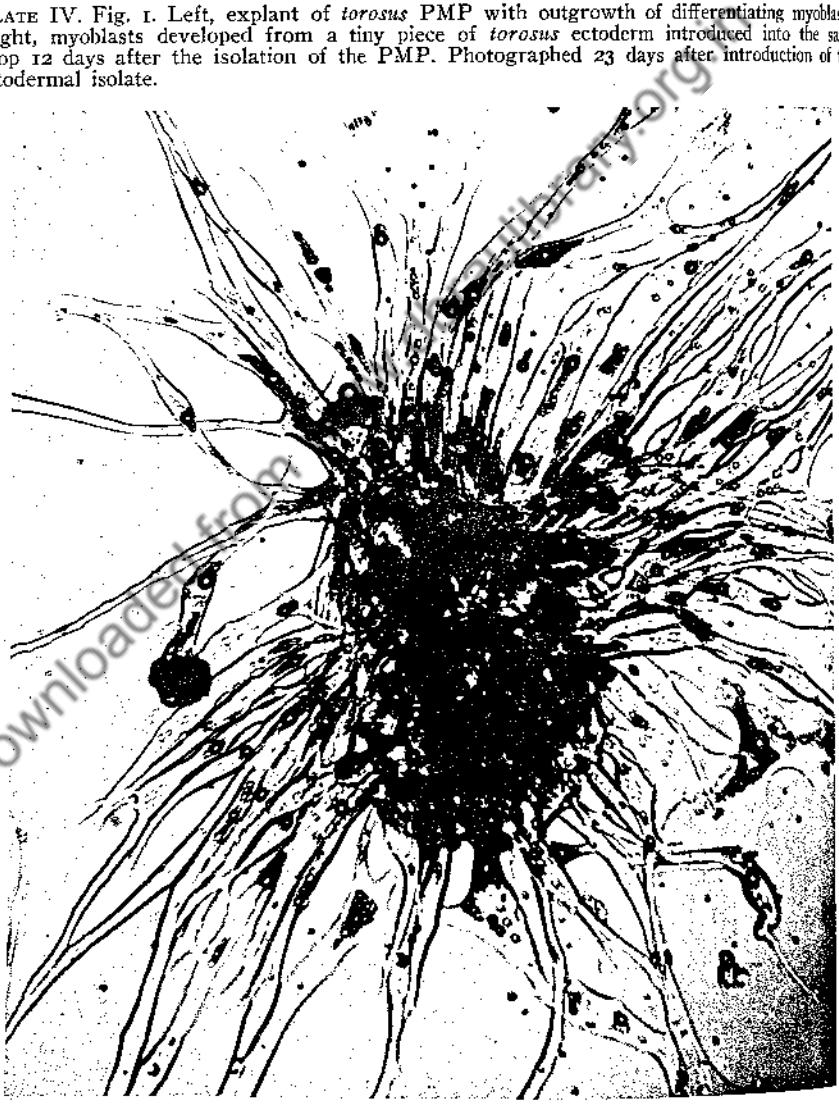


PLATE IV. Fig. 2. Myoblasts developed from a tiny piece of young *torosus* ectoderm in a 14-day old culture of *torosus* PMP. Photographed 25 days after explantation of the ectoderm.

firmed, this result would support the recent claim of Yamada and his school that protein is responsible for the ectodermal differentiation.[3]

To return to the effect of ribonuclease on induction, it can now be seen that Brachet's recent experiments (1955) prevent us from drawing any simple conclusion from the experiments mentioned above. Brachet has shown that ribonuclease may act upon ribonucleic acid within living cells. It is, therefore, possible that in our experiments the effect of ribonuclease was on the ribonucleic acid within the ectoderm cells as well as on the ribonucleic acid of the medium. Experiments are needed in which ribonuclease is removed from the medium before being used to cultivate the ectodermal cells. It is, indeed, all too apparent that the experiments on the chemical nature of the materials present in the conditioned medium are merely first steps of a projected investigation.

III. SUMMARY

1. A new technique for study of embryonic induction has been developed. It consists of explanting organizer tissue into a hanging drop of modified Holtfreter solution; 7-10 days later a small piece of young ectoderm is introduced at a distance from the developing organizer explant.

2. The inductor tissues used are the dorsal half of Spemann's organizer area, the trunk medullary plate including some adjacent neural fold, the posterior portion of the medullary plate, notochord, somitic blocks, neural folds, and endoderm of *Triturus torosus* and *Amblystoma tigrinum*. Reacting tissues are presumptive ectoderm (beginning gastrula) of *torosus, rivularis,* axolotl, and *tigrinum*.

3. The behavior of the ectodermal explant is similar to that of the explant of the neural fold. Regardless of the kind of inductor, a culture of pigment cells develops with nerve fibers radiating out of the central mass. Induction "at a distance" occurs in 90% of our experiments in our best series.

4. While young cultures (7-10 days) of PMP induce young ectoderm to differentiate as described above, older cultures (12-16 days) tend to induce formation of myoblasts (i.e. the same cell type is induced as is present in the outgrowth of the developing inductor explant). Accordingly there is more than one kind of inductor.

[3] (Added in proof.) After incubation with ribonuclease or trypsin, the conditioned medium was dialyzed and examined spectrophotometrically. It was found that the enzyme does not hydrolyze completely the ribonucleic acid or protein of the ribonucleoproteins. Accordingly, it is not possible to designate which fraction of the inductor substances is responsible for the ectodermal differentiation.

5. Young ectoderm differentiates equally well in cell-free medium withdrawn from established cultures of inductors. 24 hours' exposure to conditioned medium is sufficient to induce differentiation in the ectoderm transferred subsequently to a salt solution.

6. The conditioned medium has an absorption spectrum similar to that of a nucleoprotein. The maximal absorption of the first order medium is at 258 mμ, and of the second at 265 mμ. The significance of this difference is discussed.

7. The results of dialysis, alcohol precipitation, and color tests provide evidence that nucleoprotein is present in the conditioned medium. Enzyme studies furnish additional evidence that the nucleoprotein in the medium may be effective in induction.

BIBLIOGRAPHY

Barth, L. G. 1941. Neural differentiation without organizer. *J. Exp. Zool. 87,* 371-383.

Brachet, J. 1950. Caractéristiques biochimiques de la compétence et de l'induction. *Rev. Suisse Zool. 57,* 57-75.

Brachet, J. 1955. Action of ribonuclease and ribonucleic acid on living Amoeba. *Nature 175,* 851-853.

Caspersson, T. O. 1950. *Cell Growth and Cell Function.* W. W. Norton, New York.

Flickinger, R. E., Jr. 1949. A study of the metabolism of amphibian neural crest cells during their migration and pigmentation *in vitro*. *J. Exp. Zool. 112,* 465-484.

Grobstein, C. 1955. Tissue interaction in the morphogenesis of mouse embryonic rudiments in vitro. In *Aspects of Synthesis and Order in Growth.* Pp. 233-256. Princeton Univ. Press, Princeton.

Holtfreter, J. 1933. Nachweiss der Induktionsfaehigkeit abgetoeteter Keimteile. Isolations-und Transplantationsversuche. *Roux's Arch. 128,* 584-633.

Holtfreter, J. 1943. Properties and functions of the surface coat in amphibian embryos. *J. Exp. Zool. 93,* 251-324.

Holtfreter, J. 1944. Neural differentiation of ectoderm through exposure to saline solution. *J. Exp. Zool. 95,* 307-344.

Holtfreter, J. 1945. Neuralization and epidermization of gastrula ectoderm. *J. Exp. Zool. 98,* 161-210.

Holtfreter, J. 1947. Neural induction in explants which have passed through a sublethal cytolysis. *J. Exp. Zool. 106,* 197-222.

Holtfreter, J. 1951. Some aspects of embryonic induction. Tenth Growth Symposium, pp. 117-152.

Holtfreter, J., and V. Hamburger. 1955. Embryogenesis: progressive differentiation. In *Analysis of Development,* edited by Willier, Weiss, and Hamburger. Pp. 230-296. Saunders, Philadelphia.

McKeehan, M. S. 1951. Cytological aspects of embryonic lens induction in the chick. *J. Exp. Zool. 117,* 31-64.

Markham, R., R. E. F. Matthew, and K. M. Smith. 1948. Specific crystalline protein and nucleoprotein from plant virus having insect vectors. *Nature 162,* 88-90.

Needham, J. 1942. *Biochemistry and Morphogenesis.* Cambridge Univ. Press, London.

Needham, J. 1955. Developmental physiology. *Ann. Rev. Physiol. 17,* 37-60.

Niu, M. C. 1953. *In vitro* study of induction. *Anat. Rec. 117,* 560.

Niu, M. C. 1955. Identification of the inductor substances. *Anat. Rec. 122,* 420.

Niu, M. C., and V. C. Twitty. 1953. The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. *Proc. Nat. Acad. Sci. 39,* 985-989.

Sanford, K. K., W. R. Earle, and G. C. Likely. 1948. The growth *in vitro* of single isolated tissue cells. *J. Nat. Cancer Inst. 9,* 229-246.

Spemann, H., and H. Mangold. 1924. Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Roux's Arch. 100,* 599-638.

Tung, T. C., Y. F. Y. Tung, and C. Y. Chang. 1949. Studies on the induction of ciliary polarity in amphibia. *Proc. Zool. Soc. 118,* 1134-1179.

Twitty, V. C. 1945. The developmental analysis of specific pigment pattern. *J. Exp. Zool. 100,* 141-178.

Weiss, P. 1950. Perspectives in the field of morphogenesis. *Quart. Rev. Biol. 25,* 177-198.

Yamada, T., and K. Takata. 1955. An analysis of spino-caudal induction by the guinea-pig kidney in the isolated ectoderm of *Triturus*-gastrula. *J. Exp. Zool. 128,* 291-331.

VIII. GROWTH AND DEVELOPMENT IN THE SHOOT SYSTEM OF PLANTS

BY RALPH H. WETMORE [1]

A ROOTED plant is so much a part of its environment, both biotic and physical, that it can be studied as an entity only under controlled conditions. If conclusions concerning its continued growth, development of organs, and induction of reproductive cycle are to stand any chance of being based on factual evidence, the chosen plant must be removed from its usual association with other living beings and must be placed in a new physical environment as nearly constant as fixed variables will permit. Even then, quantitative findings on growth in the shoot system will be difficult to achieve because of photosynthesis. If one could determine that there was a fraction for plant growth which was dependent only upon incident light, other variables being constant, the quantitative dilemma could be solved. Whether one can find such a fraction is not clear at present.

To try to control the plant's environment, however, is not enough for an investigator concerned with growth and development of either the root or the shoot system of vascular plants. To make his investigations meaningful, he must become acquainted with their fundamental patterns of development and with the range of variations in these fundamental patterns under different environmental conditions.

The student of development should also take into consideration a major contrast in embryology between multicellular plants and multicellular animals. It is certainly more than a coincidence that vertebrate animals proceed to maturity through gastrulation. However varied in its steps, the end product is an animal whose absorbing and digestive system is housed within itself. Food must then be ingested. A concomitant aftermath of this method of development is the compactness and the general mobility of the mature animal organism. Attention should be called to the fact that no plant is known to reach maturity through a process of gastrulation. By contrast, the plant embryo retains the earlier,

[1] The Biological Laboratories, Harvard University. Original investigations reported on in this chapter have been supported in part by a grant from the American Cancer Society, Inc., upon recommendation by the Committee on Growth of the National Research Council, and in part by a grant from the National Science Foundation.

over-all capacity for cell division at its antipodes (Miller and Wetmore, 1945; Allen, 1947; Spurr, 1949). These continuing, actively dividing apical groups of cells or meristems of the embryo, the root and shoot meristems respectively, leave the cellular products of their mitotic activity behind them. As will be noted later, the resulting cellular accumulations are biochemically induced to differentiate by the same meristematic regions which produced them. The entity resulting from this differentiation is the plant body. Thus, as Bower (1950, and earlier) pointed out, the vascular plant has the capacity for continued embryology.

A study of the apical region, root or shoot, will always disclose histological changes accompanying differentiation of the cellular components. Studies by surgical manipulation are as revealing on the growing apical region of a tree as on a seedling of that tree. An excised apex of a half mm or less, planted *in vitro* on an adequate nutrient medium will produce an entire plant, indistinguishable from that which earlier bore the apex (Plate I, 1–6, or other examples from Ball, 1946; Wetmore and Morel, 1949; Wetmore, 1953a, 1953b).

The study of development in the entire plant is essentially the study of morphogenetic processes proximal to the root and shoot apices of that plant. While it has been clear that the plant grew by apical accretion of cells and the formation of new appendages (branch roots just proximal to the root apex, and leaves and buds at the shoot apex) little has been known of the morphogenetic processes involved. Even less has been known of the underlying physiological steps or of the biochemical pathways correlated with induction and differentiation.

If one examines the apical meristem of a fern (Plate II, 1) or an angiosperm (Plate IV, 1), one is aware of a flattened or slightly dome-shaped region (Foster, 1939, 1941; Wardlaw, 1945; Philipson, 1947, 1949; Reeve, 1948; Ball, 1949; Popham, 1951; Gifford, 1954) surrounded by leaf primordia, each of which appears in the time succession and the regular pattern characteristic of the plant. Much has been written on phyllotaxy but little experimental work has been done (Snow and Snow, 1931, 1933, 1935; R. Snow, 1942, 1951; M. Snow, 1951; Loiseau, 1951, 1954a, 1954b; Millener, 1952). Rather, consideration of leaf arrangement has been primarily mathematical, and earnest attempts have been made to reconcile the pattern of adult arrangement and even the spatial incidence of leaves in the shoot apex with the well-known Fibonacci Series (Wright, 1873; Schwendener, 1878; Church, 1904; van Iterson, 1907; Thompson, 1942; Richards, 1948, 1951). Ordi-

narily, one does not find the answer to problems raised in development by a study of the mature organism.

Six years ago, Wardlaw, at the New London, Conn., meeting of this Society (Wardlaw, 1949a), reported on some important experiments carried out in his laboratory at Manchester, England, in which, by beautifully planned knife cuts on the apical meristem of a fern, *Dryopteris aristata*, he had isolated certain actual or potential leaf primordia from the apical meristem itself and from one another (Wardlaw, 1949a, 1949b). He found that orderly development of these primordia was temporarily lost. Primordia surgically isolated from the seemingly inhibitory influences of the apical meristem itself and of their own neighboring primordia, outgrew other older primordia. From these and related studies he concluded that a new leaf primordium could originate only far enough away from the apical meristem and from the adjacent, recently formed, leaf primordia as to be subjected to less than threshold inhibition. Phyllotaxy thus comes anew into the zone of experimentation.

Two other facts, concerned with leaf and bud primordia respectively, have been claimed by Wardlaw as a result of these surgical experiments. When a region on the apex, which can be recognized by its position in relation to preceding leaves as a presumptive leaf primordium, is isolated by deep incisions from its neighboring primordia and from the apical meristem proper, that region continues its development but may appear as radially symmetrical and not as a dorsiventral organ. In other words, it tends to develop as a centric leaf, or a bud, or young branch, if one gives it its usual terminology. Sussex (1951) and more recently Cutter (1954a, 1955, 1956), Wardlaw and Cutter (1954, 1955, 1956) and Wardlaw (1955a, 1955b, 1956) have supplemented this early fern work of Wardlaw with more extensive studies. In substance, it looks as if not only the position of a leaf but even its dorsiventral symmetry is imposed upon it by the very nature of the physiological environment in the apex in which it develops. Present experimental evidence suggests that leaf and bud primordia are essentially the same initially except as influenced by the sum total of factors emanating from the adjacent apical meristem. If the meristem be isolated by a deep cut from a present or imminent leaf primordium, the latter tends to be centric. Further experiments must be carried out to extend our knowledge of this phenomenon and to determine the biochemical nature of the regulatory, hormonal substance or substances responsible for the usual dorsiventral pattern.

Sussex and Steeves (1953) and Steeves (personal communication)

in a fern (*Osmunda*) have found that leaf primordia which have achieved a certain age and position—the fifth or older—usually grew into normal dorsiventral leaves *in vitro* (Plate II, 3–5). However, any of the first five primordia when cut off and planted *in vitro* usually gave centric structures of indefinite growth which developed roots: that is, they were buds. When large enough to plant in soil, these buds became *Osmunda* plants. Steeves (unpublished) has found that older leaf primordia of an angiosperm (*Lupinus*) grew into dorsiventral leaves *in vitro*. It remains to be determined whether the youngest leaf primordia and the plaque of tissue taken from the position in which a new leaf primordium would have developed will grow *in vitro*, and, if so, whether the resulting appendages will be dorsiventral or centric.

Once an apical meristem has produced a succession of leaf primordia, it has all the potentialities of a bud. Ordinarily bud opening involves growth of leaves and elongation of the internodes, with the resultant spacing of the mature leaves on the axis according to the genetic phyllotactic pattern of the plant. Many plants, in their development and growth, achieve this internodal elongation and become regular long shoot types. By contrast, some others seem to have the leaves develop as usual, yet little or no internodal elongation occurs. It is as if the bud had opened, the leaves had matured, and then growth had stopped. Such shoots as one finds in the palms, the century plants, the dandelion, or, in their first year, some of the common vegetables like the carrot, beet, and celery, are termed short shoots. It is noteworthy that the short shoot type ordinarily develops large leaves. These two types, geologically speaking, are essentially as old as vascular plants; they are also found in almost all living groups of ferns, gymnosperms, and angiosperms. They seem to be physiological rather than evolutionary types belonging to a special subphylum or group.

When the short shoot type achieves the stature of a tree, it proves to be slow growing, e.g. according to Chamberlain (1935), a 1.5 meter cycad, *Dioon edule* of Mexico, is at least 1,000 years old; it has large leaves and weak, cylindrical stems resulting from a relatively inactive cambium layer with almost no normal wood. By contrast, long shoot trees such as most of our common trees have relatively small leaves—consider the size of a horse chestnut, a magnolia, or a maple leaf in terms of that of a tree fern or a palm—an active cambium, and much wood, the material for which we value trees.

To study further the contrasts of these short shoot-long shoot organizations, Dr. Gunckel, then of our laboratories, approached the problem

in *Ginkgo,* a tree which possesses both short and long shoots (Plate III, 1–5). Interestingly enough, a short shoot can become a long shoot, or the converse can equally be true. Part of the story proved to be readily understood. With the opening of all buds in the spring, a close correlation exists between the growth of the leaves and the amount of

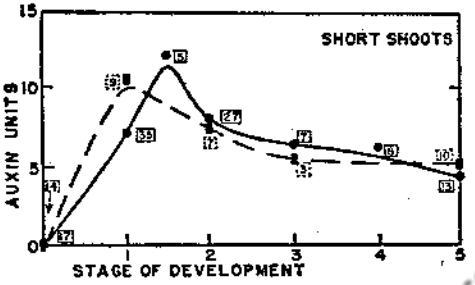


Fig. 1. Yield of diffusible auxin from short shoot buds of *Ginkgo.* Solid line: laterals. Dotted line: terminals. The data for stages 0-4 represent putative short shoots; those for stage 5 are definite. The figures in squares show the number of buds used in each determination. (Courtesy of the Editors of American Journal of Botany.)

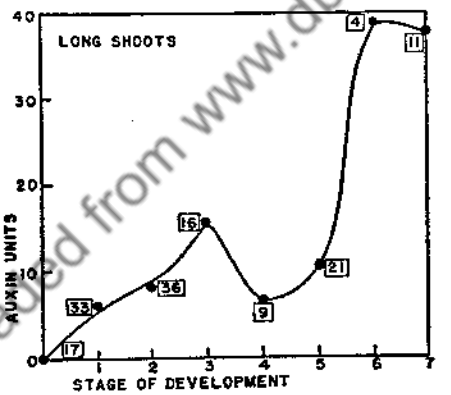


Fig. 2. Yield of diffusible auxin from long shoot lateral buds of *Ginkgo.* The data for stages 0-4 represent putative long shoots; those for stages 5-7 are definitive. The figures in squares show the number of buds used for each determination. (Courtesy of the Editors of American Journal of Botany.)

auxin available from the bud, as measured by the usual method of bioassay (Went and Thimann, 1937). With the completion of the opening process in the short shoot (Gunckel and Thimann, 1949a), the supply of auxin ceases (Fig. 1). In contrast, when a terminal bud opens, which by its position and previous pattern of growth gives evidence that it will probably produce a long shoot, the growth hormone story is quite different (Fig. 2). Though the early stages of the long shoot

provide essentially the same results as in the opening of a short shoot, the later stages are very different. Instead of a complete cessation of auxin diffusing from the bud, Gunckel found a second output which was correlated with the elongation process as the short shoot became a long shoot (Gunckel and Thimann, 1949a). Moreover, this second stage was present even when the top part of the apical meristem was removed. Accumulated evidence points to its coincidence with the rapid cell proliferation just proximal to the meristem proper, and suggests that, at least in *Ginkgo,* internodal elongation can be correlated with a net production of auxin which moves downward in polarized fashion towards the region of elongation, where presumably its concentration is raised to the level conducive to cell growth.

As one might expect, upon removal of a terminal bud and therefore of lateral shoot inhibition, one or more lateral short shoots immediately below the amputated stump (Plate III, 3) tend to become long shoots (Gunckel, Thimann, and Wetmore, 1949b). The auxin background for this type of change is in conformity with known facts, even though bud inhibition in correlative growth is not fully understood. The converse of this phenomenon, why terminal long shoots become short, is less clear. Certainly other factors than the auxin relations are involved.

For the present, it can be said that certain other experimental studies have provided facts as significant in understanding part of the long shoot-short shoot story as those known from the studies on *Ginkgo* (Titman and Wetmore, 1955; Steeves and Wetmore, 1953). Correlations between the size of the leaf, failure to develop a significant cambium with characteristic pattern and the insignificant seasonal increment of xylem and phloem are not understood (Plate III, 6 and 7; cf. Seward, 1919; Titman and Wetmore, 1955). One should realize perhaps that the ordinary flower of an angiosperm or the cones of a pine tree are essentially contrivances of the short shoot type. An understanding of the mechanisms underlying short shoot formation may help elucidate the still unanswered questions of photoperiodism and the induction of buds which become floral short shoots from the same apical meristem which previously gave vegetative long shoots.

In this connection I want to comment in some detail on the organization of apical meristems. The apical meristem in most ferns (Plate II, 1) consists of a single layer of cells. In the center of this layer is an apical cell, somewhat larger than its derivative flanking cells. Longitudinal divisions are infrequent in this layer, except around the periphery where leaf and bud primordia, as indicated earlier, have their

origins in regions of low inhibition. Beneath this single layer, there is generally found a cap of potential vascular tissue (Plate II, 1; Fig. 3), confluent on the flanks with that of the stem and of the leaves (Ward-

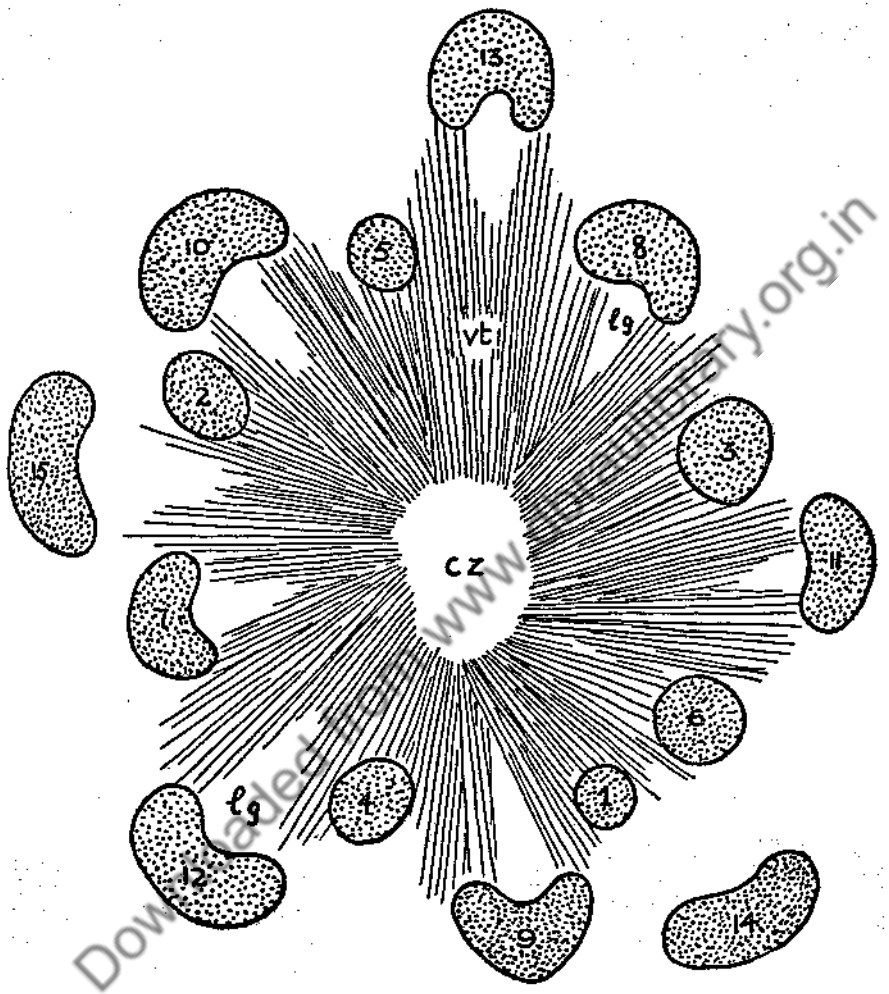


Fig. 3. Apical view of cap of prestelar tissue underlying the apical meristem of *Osmunda cinnamomea*, as seen in section in Plate II, 1. The gaps confronting the individual leaves are already obvious. Courtesy of Professor Taylor A. Steeves.

law, 1944-1947). This cap may cover a complete cylinder of vascular tissue in the stem behind it (Plate I, 1; Plate II, 2) or the cells comprising its central part may undergo frequent divisions in a plant at right angles to the axis, so forming a pith or medulla of rows of parenchymatous cells, occupying the whole central region of the vascular

cylinder (Plate IV, 1). This region of cells characteristically dividing in a single plane is termed a *rib meristem.*

One could show a variety of other histological variants in the organizational pattern of apical meristems. I want to mention only one particularly, one which I believe is truly different in what it represents. The apical meristem of an angiosperm, cut in median plane, e.g. *Helian-*

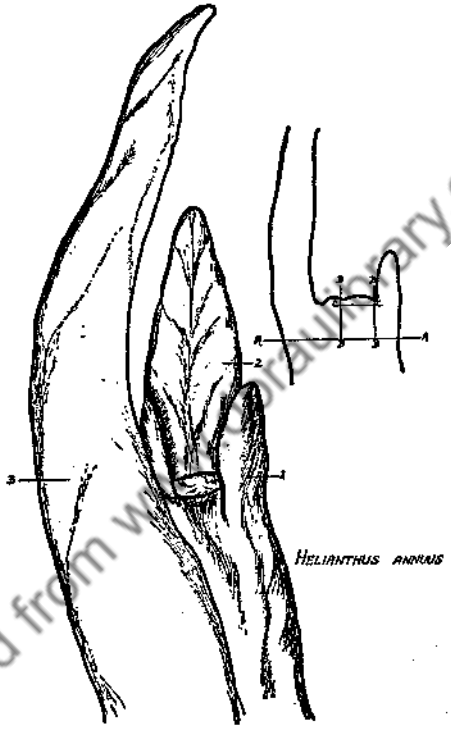


Fig. 4. Dissection of center apical bud of *Helianthus annuus,* showing the three youngest leaf primordia around the apical meristem. The drawing on the upper right indicates how incisions are made in removing the whole apex (cut AA), the flanking portions (BB), and the central core. The small apical piece denoted by CC has no significance in the present legend in this study. (Drawn by Miss Barbara Donahue).

thus annuus, shows a central portion of large, vacuolated cells, quite different from the surrounding sheath of small, densely cytoplasmic cells flanking this central portion. From the outer sheath originate the leaves and the buds. The rib meristem at the base of the apical meristem gives the pith.

It can be stated that a piece of an apical meristem of *Helianthus annuus* (Plate IV, 1) from the tip of the axis of a bud and deep enough to include the pith rib meristem (Fig. 4, that part above cut AA), that

is, about 0.5 mm or so deep, will, on a suitable medium, grow into a whole new plant (Plate IV, 4). Such a medium of synthetic nature consists of mineral salts, minor elements, an auxin in physiological concentration (e.g. 0.05 mg per l of indole acetic acid is average for many plants tried), 15% coconut milk, and 1 mg per l of vitamin-free casein hydrolysate.

If the flanks are cut from the apical meristem of *Helianthus annuus* so as to exclude the central core or pedestal of large cells (that is, lateral to cuts BB, Fig. 4), and if these flanks are planted *in vitro*, they characteristically grow (Plate IV, 2). Ordinarily each explant produces a leaf, if a leaf primordium were originally included, the basal tissue giving a shapeless mass of cells termed callus. Not infrequently this growing explant will root. In no case has a whole plant resulted from such a culture but this may yet happen.

If the excised central core (that part between the two cuts marked BB on Fig. 4), consisting mostly of enlarged cells, is planted on the same medium as were the whole apices and the flanks, essentially no growth occurs (Plate IV, 3), except that the rib meristem at the base, if present, may produce a mass of unorganized callus.

When one examines histological preparations of the whole apex under conditions favorable to growth, cell divisions prove frequent in the peripheral regions, and scarce, or often absent, in the large-celled central region (Plate IV, 1). On the face of it, this would seem to be contradictory to expectation from an apical meristem.

Recent experience with three species of plants:—*Chenopodium album* (lamb's quarters), *Xanthium saccharatum* (cocklebur) and *Glycine max* (Biloxi soybean), with known photoperiods,[2] indicates that towards the close of the induction period, buds killed, fixed, and sectioned show the large-celled central region to be cut up into small, actively dividing cells. In other words, as the vegetative bud is induced to become a flowering bud, the latent central part of the vegetative bud is activated and contributes to the inflorescence and flower production. Our work has not yet progressed far enough to permit me to say whether there is a definite contribution to any particular part of the reproductive axis from that portion of the apical meristem. If it is found that a similar situation exists after investigating more angiosperms with varying patterns of organization in their apical meristems and with different induction periods, one may be in a better position to interpret the zonation of the

[2] Acknowledgement is made to Dr. H. A. Borthwick of the United States Department of Agriculture for seeds of various species of plants used in this study.

angiospermous apex. If one were rash enough to extrapolate beyond the existing facts, one might hazard a statement that there is in the center of the angiospermous apex, and perhaps in the apices of similarly organized higher gymnosperms as well, a column of tissue which takes part in the reproductive phase of the life history only, a kind of Weismann's Germ Plasm.

I had hoped to be able to state whether the apex planted *in vitro* can be induced by exposing it alone at least to the requisite number of short days needed for the induction of the whole plant. However the *in vitro* growth of the apices of these three species so exposed has not as yet produced flowers. In any case, the change in topography from the more or less flat vegetative apex to the somewhat rounded flowering apex in the angiosperm can now be understood in three species at least as reflecting activity in the central part of the apex.

Any consideration of morphogenesis in the vascular plants involves a consideration of organization, that is, of fundamental pattern. Throughout the entire groups of vascular plants, one finds a common pattern as fundamental as that in the vertebrate animals. A vascular system forms a general central cylinder in all types, root and shoot. This central cylinder may have a parenchymatous pith up the center, or it may not; it may have it in the stem but not in the root; it may have it in adult form but not when young; it may have it in its horizontal, underground rhizome but not in its upright aerial stem; it may have it when young but it may be completely broken up into separate, scattered strands when mature; and so on with variations. This vascular cylinder is characteristically surrounded by a cortex and the whole covered with an epidermis. The epidermis may have stomata associated with aeration or it may be aerated through the leaves only as in many Conifers, Lycopodia, etc. So long as the plant has no cambium, that is, has a primary organization only, this is a fundamental pattern. Few efforts have been made to inquire into the factors underlying the differentiation of its tissues in this pattern.

It is clear that the amputation of the apical meristem from an axis of the shoot, main or branch, completely stops the differentiation in that axis. Torrey (oral communication at A.I.B.S. meeting, 1954) has recently shown that the same situation prevails in the root.

Jacobs (1952, 1954) has reviewed the earlier work concerned with the differentiation of vascular tissues. He has also reported on some clean-cut experiments which have led him to believe that the differentia-

tion of xylem is conditioned by at least two recognized variables, namely, an adequate carbohydrate supply and an auxin in physiological concentration. In his studies (1952), Jacobs found that when a sizable V-shaped cut was made in an internode of *Coleus* below a leaf, any resulting discontinuity in the vascular tissue was repaired by the formation of new xylem cells from mature pith cells in a basipetal direction around the wound. If the leaf was cut off, no such generation of xylem from pith tissues occurred unless the cut stump of the petiole was covered with lanolin containing auxin. In these instances, it was clear that auxin was a limiting factor and, even more, that the critical amount of auxin was that which was *available* at the cut area and not the amount *supplied*. In other words, Jacobs stressed the fact that the transport of auxin to the using area was more important than the absolute amount available at the source.

In our own studies, significant results have been obtained from apex-callus grafts (Plate V, 1–3). Callus of ordinarily homogeneous parenchyma obtained from the cambial region of lilac has been grown in our laboratories for close to six years. In the course of our studies it became apparent that if an apex of lilac was grafted into lilac callus, the graft being facilitated by filling the V-shaped cut with agar, the graft union was effected by an interdigitation resulting from growth on the part of both scion and callus host (Plate V, 4; Wetmore and Sorokin, 1955). The important thing, however, was that in all cases of active growth of the scion, an induction of vascular tissue took place in the otherwise homogeneous callus (Contrast Plate V, 6 and 7 with vascular tissue, and 5 without) (Wetmore, 1953; Wetmore and Sorokin, 1955). This vascular tissue developed in separate strands in basipetal fashion in that part of the callus close to and immediately below the grafted scion (Fig. 5). If nutrient agar containing auxin was used to fill the cut, the vascular strands appeared as before but at varying distances from the graft union (Fig. 6), the distances dependent upon the concentration of the auxin in the agar.

With this evidence in hand, experiments were tried in which no scion was used and only agar containing auxin employed in the cut. In these instances again vascular tissue appeared, the disposition of the separate strands depending upon the concentration of the auxin in the cut (Plate V, 6 and 7), all concentrations employed being within the physiological range. Since sucrose was used in the medium, it appears that auxin was limiting, and that, within the range of concentrations found

in shoot systems of plants, vascular tissues could be induced in otherwise homogeneous parenchymatous tissue at almost predictable positions.

It is important to point out that thus far this induced vascular tissue has been comprised of xylem when mature, the elements being vessels with oblique, porous perforations in the end walls and crowded, circular-bordered pits in the side walls, as is characteristic of *Syringa*. The elements are shorter, however, than in the plant and they are not aggregated in the continuous vascular cylinder found in lilac.

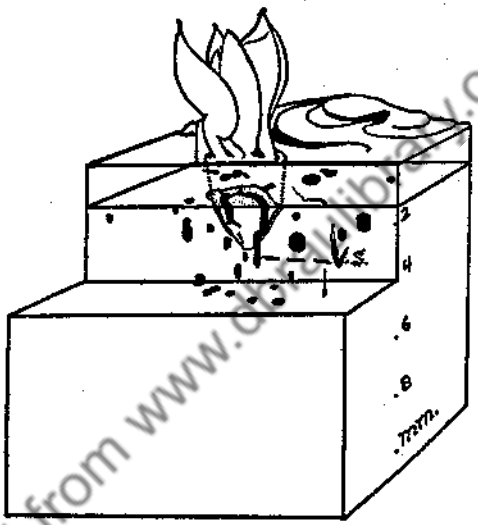


Fig. 5. Stereogram of a grafted apex of *Syringa* into *Syringa* callus showing the disposition of the vascular strands in relation to the graft union. The incision was filled with 1% agar alone. Note the near continuity of vascular tissue of the scion and a strand in the callus immediately below. Note the scattered strands also in the proximity of the graft union. (Drawn from serial sections by Mr. Sergei Sorokin. Published with the permission of the Editors of the Journal of the Arnold Arboretum.)

Among other observations that may be cited at this time, the first is that no phloem has been found in its naturally almost universal association with xylem. Our evidence, and seemingly that implicit in the studies of Jacobs (1952, 1954) and Sinnott and Bloch (1944), suggests that the limiting factors controlling the differentiation of xylem and phloem are independent. The second observation stresses the necessity of recognizing that the position of the xylem at least is controllable and is influenced by the same factor which limits its appearance.

One can question whether a first approach to the problem of the

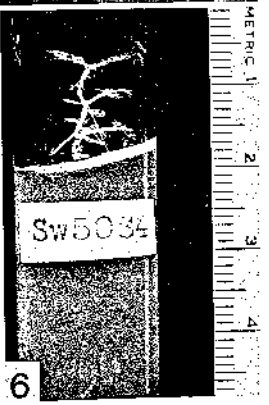


PLATE I. 1. Median longitudinal section of stem apex of *Selaginella rupestris*. Note apical cell, solid core of vascular tissue with cells in varying stages of differentiation. ×164. 2. Cultures on sterile mineral salt-sucrose agar of terminal 0.5 mm piece of apex of *Selaginella willdenovii* after 9 weeks. ×1.5. 3. Similar cultures after 11 weeks. ×¾. 4. Similar cultures after 8 weeks, showing normal leaf size and distribution for this anisophyllous species. ×1.5. 5. Plant of *S. willdenovii* now growing in greenhouse at Harvard University. Initially planted as above *in vitro* from a small piece of stem apex, grown *in vitro* for 6.5 months, then planted in soil. Photograph taken 10 months later. ×1/7. 6. Similar culture after 12 weeks. Note rhizophores on stem from the basal ends of which roots develop. ×¾.

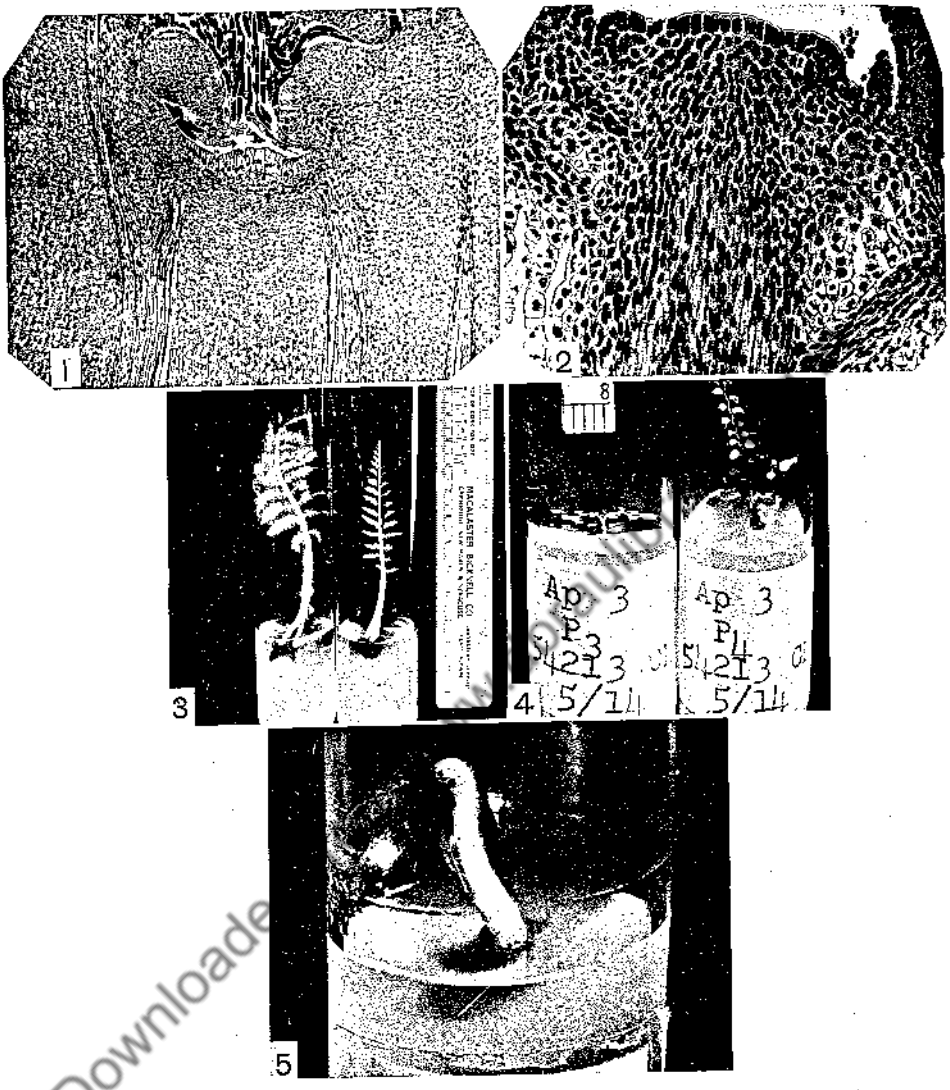


PLATE II. 1. Median longitudinal section of cinnamon fern, *Osmunda cinnamomea*. Note centrally placed apical meristem with layers of somewhat transversely elongate prestelar tissue below it continuous with strands of differentiating vascular tissue on the flanks. ×35. 2. Median longitudinal section of apex of *Lycopodium selago*. Note central column of vascular tissue extending within two cells of outermost layer of meristem. Some differentiation is shown in lower part of vascular column. ×105. 3. Cultures of leaf primordia of cinnamon fern on simple mineral salt—sucrose medium, after 3 months. ×⅓. 4. Culture of third and fourth leaf primordia of maidenhair fern, *Adiantum pedatum*, on same medium after about 5 weeks. ×⅓. 5. Culture of leaf primordium of cinnamon fern after 5 weeks. ×⅓. (I am indebted to Professor T. A. Steeves for the privilege of using Figs. 1, 3, 4, and 5. Acknowledgment for Fig. 2 is made to the Editors of Torreya in which the author had previously used this figure.)

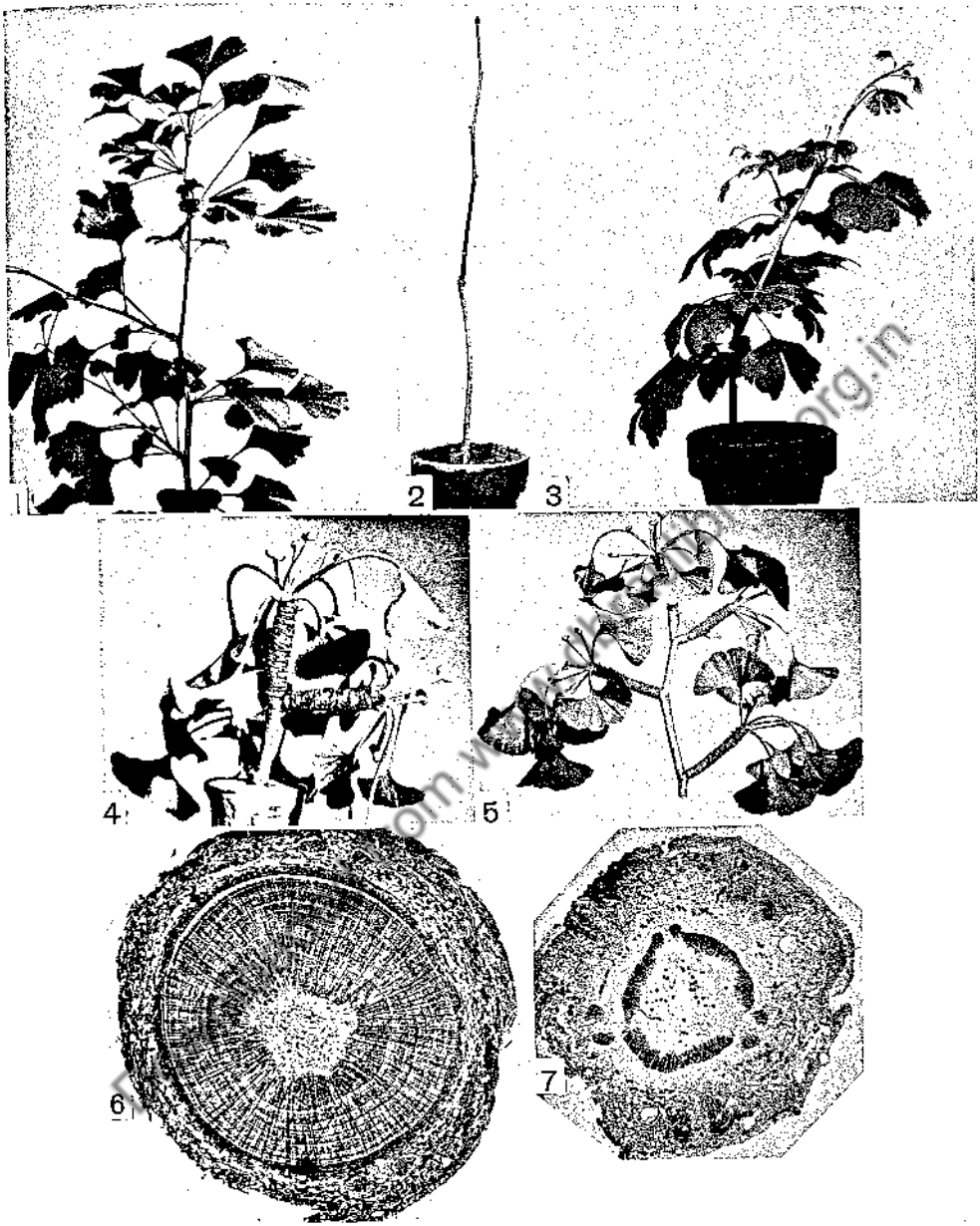


PLATE III. 1, 2, and 3. Long shoot growth in *Ginkgo biloba*, with lateral short shoots. Note (1) two lateral long shoots and terminal short shoot. 4 and 5. Short shoot habits in *Ginkgo*, both terminal and lateral. Note branches bearing ovules in both. 6. Transverse section of long shoot, 8 years old. Note large amount of xylem produced by an active cambium. ×6.5. 7. Transverse section of an old short shoot, though age is unknown. Note little xylem, characteristic of the much less active cambium of a short shoot. ×6.5. (Professor J. E. Gunckel has provided negatives for 1–5 for which the author is grateful.)

disposition of vascular tissues in the axes of plants is at hand. Certainly there is little insight as yet into the development and differentiation of tissues generally in the vascular plants, but a direction for experimental work and possible techniques to initiate further studies seem evident.

Any attempt at an over-all view of growth and development in higher plants and any interpretation of differentiation of cells and tissues in

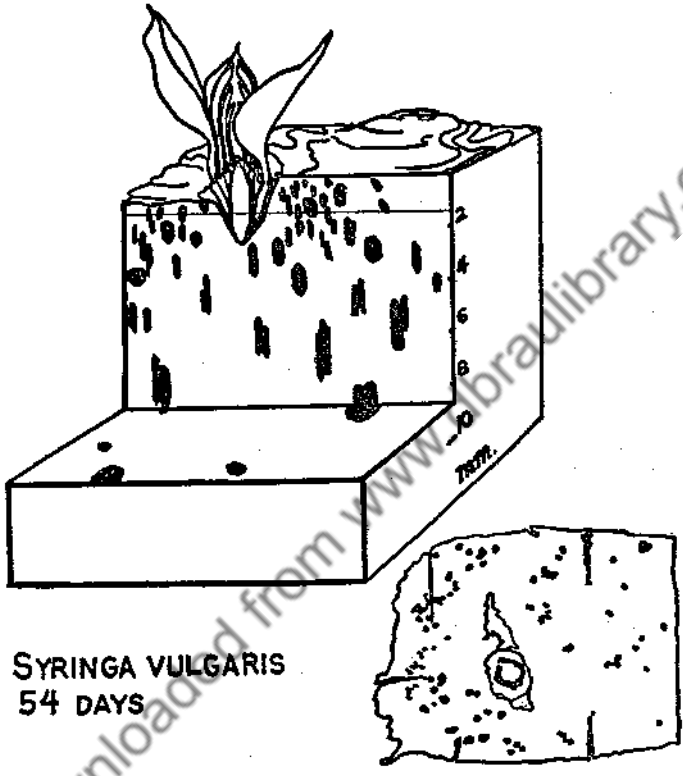


Fig. 6. Stereogram of a graft similar to that of Fig. 5 except that the incision was filled with 1% agar containing 0.05 mg/l. naphthalene acetic acid. Transverse section below is from the 2 mm level as indicated. (Drawn by Mr. Sergei Sorokin. Published with the permission of the Editors of the Journal of the Arnold Arboretum.)

the development of organs as parts of these plants must first recognize the plant as a generalized organism. It has no vascular circulation, no nerves, no muscles, no endocrine glands. A vascular plant grows continuously at its apices except as limited by its external environment, or by reproduction which utilizes its apical meristems, or by accident or disease. The organism is never mature unless its continued embryology is closed by one of the above events. It is true that parts of it may be

mature in that the component cells may have acquired a seemingly permanent, functional state with a characteristic cytology and function. But it is questionable whether any living plant cell, possessed of a nucleus, is irreversibly differentiated. Epidermal cells may take part in the formation of a cork cambium (Eames and MacDaniels, 1947) or produce a bud (Naylor and Johnson, 1937). Collenchyma cells with their irregularly cellulose-thickened walls may divide and become part of a cork cambium. Even thick-walled parenchyma cells in the xylem, produced by the vascular cambium a number of years earlier, may have their protoplasts grow, push out of their lignified, cellulose strait jackets through pits or pit-pairs into adjacent dead vessels or intercellular spaces and, if a nucleus happens to leave an original cell, the extruded partial protoplast is said to undergo mitosis. When divided, the daughter cells may lay down their own secondary walls and become pitted (Eames and MacDaniels, 1947). Adventitious buds may generate from almost any topographical part of a plant, the cells of which may have been considered already differentiated. Even evanescent organs like the petals of a flower have been known to bud when they have fallen on moist earth and the environment remained favorable. I am probably not overstating my case if I suggest that all plant cells are capable of growth and/or cell division, unless they be dead like the conducting or fibrous cells of the xylem, or non-nucleated like the sieve members of the phloem, or suberized like the cells of bark. Here then differentiation can only connote change, and this may be or need not be followed by further change. If the change is in the direction of the production of a meristematic group of cells, it could without concern be spoken of as dedifferentiation.

Normally, the apical meristems add new cells, give rise to new appendages, and effectively control the differentiation of vascular tissues. If the initial short shoot of the bud becomes a long shoot, the internodal elongation seems independent of the apical meristem; such evidence as we have indicates that an adequate concentration of the growth hormone necessary to promote this elongation is achieved in part from the young leaves and in part from the actively dividing portions of the internodes themselves.

Much evidence is in hand to indicate that the natural auxins are hormonal in promoting growth and must be considered as humoral in that there are no endocrine glands and their availability is general, especially as they move in a polarized direction. Effective concentrations may depend upon (1) availability, (2) antiauxins or inhibitors,

and (3) inactivating mechanisms. While chromatography of auxins has now shown (Bennett-Clark and Kefford, 1953; Stowe and Thimann, 1954; Sen and Leopold, 1954; Linser et al., 1954; Fischer, 1954; Nitsch and Nitsch, 1955) that a family of compounds, not a single auxin, seems to be available in any plant material or tissue investigated, too little is yet known to justify statements on any specific roles each might play in growth or differentiation within the plant. The contributions to many processes which can be ascribed to auxins suggest that the problems to be solved may be no less difficult than those presented to students of animal hormones.

The present program of research, certain aspects of which are reported on here, has suggested some new approaches, or new tools conceivably, for tackling old problems. However, the surface has only been scratched. Perhaps the major contribution of this research has been to teach those involved in it that many problems of morphogenetic nature can be attacked better on the vascular cryptogams than on the angiosperms. The zoologist has long since employed the approach of doing fundamental developmental studies on the lower groups; the botanist may well learn it.

BIBLIOGRAPHY

Allen, G. S. 1947. Embryogeny and development of the apical meristems of *Pseudotsuga*. III. Development of the apical meristems. *Amer. Jour. Bot. 34*, 204-211.

Ball, E. 1946. Development in sterile culture of stem tips and subjacent regions of *Tropaeolum majus* L. and *Lupinus albus* L. *Amer. Jour. Bot. 33*, 301-318.

Ball, E. 1949. The shoot apex and normal plant of *Lupinus albus* L., bases for experimental morphology. *Amer. Jour. Bot. 36*, 440-454.

Bennet-Clark, T. A., and N. P. Kefford. 1953. Chromatography of the growth substances in plant extracts. *Nature 171*, 645.

Bower, F. O. 1950. *Botany of the Living Plant*. 4th ed. MacMillan, London.

Buvat, R. 1952. Structure, évolution et fonctionnement du méristème apical de quelques Dicotylédones. *Ann. Sci. Nat. Bot. 11e sér., 13*, 199-300.

Chamberlain, C. J. 1935. *Gymnosperms, Structure and Evolution*. Univ. of Chicago Press, Chicago.

Church, A. H. 1904. *On the Relation of Phyllotaxis to Mechanical Laws*. Williams and Norgate, London.

Cutter, E. G. 1954. Experimental induction of buds from fern leaf primordia. *Nature 173*, 440-444.

Cutter, E. G. 1955. Experimental and analytical studies of Pteridophytes. XXIX. The effect of progressive starvation on the growth and or-

ganization of the shoot apex of *Dryopteris aristata* Druce. *Ann. Bot. 19,* 485-499.

Cutter, E. G. 1956. Experimental and analytical studies of Pteridophytes. XXXIII. The experimental induction of buds from leaf primordia in *Dryopteris aristata* Druce. *Ann. Bot. 20,* 143-165.

Eames, A. J., and L. H. MacDaniels. 1947. *An Introduction to Plant Anatomy,* 2nd ed. McGraw-Hill, New York.

Fischer, A. 1954. Über die papierchromatographische und papierelectrophoretische Trennung von Indolederivaten. *Planta 43,* 288-314.

Foster, A. S. 1939. Problems of structure, growth and evolution in the shoot apex of seed plants. *Bot. Rev. 5,* 454-470.

Foster, A. S. 1941. Comparative studies on the structure of the shoot apex in seed plants. *Bull. Torrey Bot. Club 68,* 339-350.

Gifford, E. M. 1954. The shoot apex in angiosperms. *Bot. Rev. 20,* 477-529.

Gunckel, J. E., and K. V. Thimann. 1949a. Studies of development in long shoots and short shoots of *Ginkgo biloba* L. III. Auxin production in shoot growth. *Amer. Jour. Bot. 36,* 145-151.

Gunckel, J. E., K. V. Thimann, and R. H. Wetmore. 1949b. Studies of development in long shoots and short shoots of *Ginkgo biloba* L. IV. Growth habit, shoot expression and the mechanism of its control. *Amer. Jour. Bot. 36,* 309-318.

Jacobs, W. P. 1952. The role of auxin in differentiation of xylem around a wound. *Amer. Jour. Bot. 39,* 301-309.

Jacobs, W. P. 1954. Acropetal auxin transport and xylem regeneration—a quantitative study. *Amer. Nat. 88,* 327-337.

Johnson, M. A. 1951. The shoot apex in gymnosperms. *Phytomorphology 1,* 188-204.

Lance, A. 1952. Sur la structure et le fonctionnement du point végétatif de *Vicia faba* L. *Ann. Sci. Nat. 11e sér., 13,* 301-339.

Linser, H., H. Mary, and T. Maschek. 1954. Papierchromatographie von zellstreckend wirksamen Indolkorpern aus *Brassica*-Arten. *Planta 44,* 103-120.

Loiseau, J. E. 1954a. Suppression expérimentale d'une hélice foliare chez *Impatiens Roylei* Walp. *Comp. Rend. Acad. Sci. 238,* 149-151.

Loiseau, J. E. 1954b. Evolution morphologique de quelques tiges d'*Impatiens Roylei* Walp. après suppression expérimentale d'une hélice foliaire. *Compt. Rend. Acad. Sci. 238,* 385-387.

Millener, L. H. 1952. An experimental demonstration of the dependence of phyllotaxis on rate of growth. *Nature 169,* 1052-1053.

Miller, H. A., and R. H. Wetmore. 1945. Studies in the developmental anatomy of *Phlox drummondii* Hook. I. The embryo. *Amer. Jour. Bot. 32,* 588-599.

Naylor, E. E., and B. Johnson. 1937. A histological study of vegetative reproduction in *Saintpaulia ionantha. Amer. Jour. Bot. 24,* 673-678.

Nitsch, J. P., and C. Nitsch. 1955. The separation of natural plant growth substances by paper chromatography. *Beitr. z. Biol. der Pflanzen 31,* 387-408.

Philipson, W. R. 1947. Some observations on the apical meristems of leafy and flowering shoots. *Jour. Linnean Soc. London, Bot. 53,* 187-193.

Philipson, W. R. 1949. Ontogeny of the shoot apex in dicotyledons. *Biol. Rev. Cambridge Phil. Soc. 24,* 21-50.

Popham, R. A. 1951. Principal types of vegetative shoot apex organization in vascular plants. *Ohio Jour. Sci. 51,* 249-270.

Reeve, R. M. 1948. The "tunica-corpus" concept and development of shoot apices in certain dicotyledons. *Amer. Jour. Bot. 35,* 65-75.

Richards, F. J. 1948. The geometry of phyllotaxis and its origin. *Symposia, Soc. Exper. Biol. 2,* 217-245.

Richards, F. J. 1951. Phyllotaxis: its quantitative expression and relation to growth in the apex. *Phil. Trans. Roy. Soc. London, B, 235,* 509-564.

Schwendener, S. 1878. *Mechanische Theorie der Blattstellungen.* W. Engelmann, Leipzig.

Sen, S. P., and A. C. Leopold. 1954. Paper chromatography of plant growth regulators and allied compounds. *Physiol. Plantarum 7,* 98-108.

Seward, A. C. 1919. *Fossil plants. IV. Ginkgoales, Coniferales, Gnetales.* Cambridge Univ. Press, London.

Sinnott, E. W., and R. Bloch. 1944. Visible expression of cytoplasmic pattern in the differentiation of xylem strands. *Proc. Nat. Acad. Sci. 30,* 388-392.

Snow, M. 1951. Experiments on spirodistichous shoot apices. I. *Phil. Trans. Roy. Soc. London, B, 235,* 131-162.

Snow, M., and R. Snow. 1931. Experiments on phyllotaxis. I. The effect of isolating a primordium. *Phil. Trans. Roy. Soc. London, B, 221,* 1-43.

Snow, M, and R. Snow. 1933. Experiments on phyllotaxis. II. The effect of displacing a primordium. *Phil. Trans. Roy. Soc. London, B, 222,* 353-400.

Snow, M., and R. Snow. 1935. Experiments on phyllotaxis. III. Diagonal splits through decussate apices. *Phil. Trans. Roy. Soc. London, B, 225,* 63-94.

Snow, R. 1942. Further experiments on whorled phyllotaxis. *New Phytol. 41,* 108-124.

Snow, R. 1951. Experiments on bijugate apices. *Phil. Trans. Roy. Soc. London, B, 235,* 291-310.

Spurr, A. R. 1949. Histogenesis and organization of the embryo in *Pinus strobus* L. *Amer. Jour. Bot. 36,* 629-641.

Steeves, T. A., and R. H. Wetmore. 1953. Morphogenetic studies in *Osmunda cinnamomea* L. Some aspects of the general morphology. *Phytomorphology 3,* 339-354.

Stowe, B. B., and K. V. Thimann. 1954. The paper chromatography of indole compounds and some indole-containing auxins of plant tissues. *Arch. Biochem. Biophys. 51,* 499-516.

Sussex, I. M. 1951. Experiments on the cause of dorsiventrality in leaves. *Nature 167,* 651-654.

Sussex, I. M., and T. A. Steeves. 1953. Growth of excised fern leaves in sterile conditions. *Nature 172,* 624-627.

Thompson, D. W. 1942. *On Growth and Form,* 2nd ed. Cambridge Univ. Press, London.

Titman, P. W., and R. H. Wetmore. 1955. The growth of long and short shoots in *Cercidiphyllum. Amer. Jour. Bot. 42,* 364-372.

Van Iterson, G. 1907. *Mathematische und mikroscopisch-anatomische Studien über Blattstellungen.* G. Fisher, Jena.

Wardlaw, C. W. 1944-1947. Experimental and analytical studies of Pteridophytes. Parts 3 to 10. *Ann. Bot.* 1944–1947.

Wardlaw, C. W. 1945. The shoot apex in Pteridophytes. *Biol. Rev. Cambridge Phil. Soc. 20,* 100-114.

Wardlaw, C. W. 1949a. Experiments on organogenesis in ferns. *Growth 9,* 93-132.

Wardlaw, C. W. 1949b. Phyllotaxis and organogenesis in ferns. *Nature 161,* 167-174.

Wardlaw, C. W. 1955a. Experimental investigation of leaf formation, symmetry and orientation in ferns. *Nature 175,* 115-121.

Wardlaw, C. W. 1955b. Experimental and analytical studies of Pteridophytes. XXVIII. Leaf symmetry and orientation in ferns. *Ann. Bot. 19,* 390-399.

Wardlaw, C. W. 1956. Experimental and analytical studies of Pteridophytes. XXXII. Further investigations on the effect of undercutting fern leaf primordia. *Ann. Bot. 20,* 121-132.

Wardlaw, C. W., and E. G. Cutter. 1954. Effect of deep and shallow incisions on organogenesis at the fern apex. *Nature 174,* 734-737.

Wardlaw, C. W., and E. G. Cutter. 1955. Experimental and analytical studies of Pteridophytes. XXX. Further investigation of the formation of buds and leaves in *Dryopteris aristata* Druce. *Ann. Bot. 19,* 515-526.

Wardlaw, C. W., and E. G. Cutter. 1956. Experimental and analytical studies of Pteridophytes. XXXI. The effect of shallow incisions on organogenesis in *Dryopteris aristata* Druce. *Ann. Bot. 20,* 39-56.

Went, F. W., and K. V. Thimann. 1937. *Phytohormones.* Macmillan, New York.

Wetmore, R. H. 1953a. Tissue and organ culture as a tool for studies in development. *Proc. 7th Internat. Bot. Congr. (Stockholm),* 369-370. 1950.

Wetmore, R. H. 1953b. The use of "in vitro" cultures in the investigation of growth and differentiation in vascular plants. *Brookhaven Symposia in Biol. No. 6,* 22-40.

Wetmore, R. H., and G. Morel. 1949. Growth and development of *Adiantum pedatum* L. on nutrient agar. *Amer. Jour. Bot. 36,* 805-806.

Wetmore, R. H., and S. Sorokin. 1955. On the differentiation of xylem. *Jour. Arnold Arb. 36,* 305-317.

Wright, C. 1873. The uses and origin of the arrangement of leaves in plants. *Mem. Amer. Acad. Arts and Sci. 9,* 379-415.

IX. DEVELOPMENTAL CORRELATIONS IN NEUROGENESIS

BY VIKTOR HAMBURGER [1]

THE foundation of experimental neurogenesis was laid forty years ago by Harrison and his associates, notably, Detwiler. Three major problems emerged from their pioneer studies: The determination of the basic structural pattern of the central nervous system; the developmental correlations, both intracentrally and between nervous and non-nervous structures, which are instrumental in its elaboration; and the determination of peripheral nerve patterns. The last-mentioned problem came into focus after the controversy over the origin of the nerve fibers had been definitely settled by the classical tissue culture experiment of Harrison (1907). This problem will not be discussed here because it has been a topic in two previous Symposia: its broader aspects have been dealt with by Weiss (1941), and the selective affinities between nerve endings and their specific partners, by Sperry (1951). I shall concentrate on the problem of *correlations,* but a brief reference to the determination of the basic structure is necessary as a background for the discussion of these correlations. (For recent reviews see Hamburger and Levi-Montalcini, 1950; Hamburger, 1952; and Weiss, 1955.)

I. DETERMINATION OF THE BASIC PATTERN OF THE CENTRAL NERVOUS SYSTEM

As is well known, the origin of the nervous system can be traced back to the inductive activity of the archenteron roof. Regional differences in the inductivity of the archenteron roof account for the blocking out of the major subdivisions of the central nervous system, the archencephalon, deuterencephalon, and spinal cord, but the steps immediately following the induction seem to be brought about by "self-organization" processes within the primordium. The early neural tube of the chick embryo is already a highly organized system: a detailed topographic pattern of substructures is laid out along the main axis, and the dorsoventral strati-

[1] Washington University, St. Louis. The investigations from this laboratory are supported by a research grant (PHS B-463) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service, and by a grant-in-aid from the National Cancer Society, upon recommendation of the Committee on Growth of the National Research Council.

fication is established in the spinal cord (see Hamburger, 1952). This information was obtained from transplantation and defect experiments which reveal differences before they can be detected otherwise. But these experiments have their definite limitations: They deal with relatively large units and leave the question of the determination of fine structure unanswered. We do not know when and how the cytological specifications of the different cell strains such as motor, internuncial, and glia are established. Our ignorance with respect to this point is largely due to the difficulties involved in designing the appropriate experiments. It would be necessary to trace and handle individual cells and small cell groups, for which we are not yet prepared. On the other hand, we are aware that we have to deal with these problems of differentiation simultaneously on two levels of organization, the cellular and the supercellular —thus adding another complication. Fortunately, there are redeeming features in the picture. The central nervous system remains a relatively simple tube which undergoes no complex morphogenetic changes. By and large, neurogenesis follows a rather regular sequence of steps which, though overlapping, can be analyzed separately: (1) the proliferative activity which is localized near the lumen of the central canal; (2) migration and mantle formation; (3) initial differentiation and fiber formation during or after this migration; (4) the formation of cell columns and nuclei by grouping and segregation of cell clusters, occasionally accompanied or followed by a second migration; (5) extensive cell and axon growth, and finally (6) the establishment of synaptic connections.

Proliferation does not serve merely as a device for the formation of building stones. The distribution of mitoses was found to be not at random but patterned in space and time and related to the subsequent migration, differentiation, and localization processes. This relationship was brought out in studies on the spinal cord of Amphibian (Coghill, 1933) and chick embryos (Hamburger, 1948). More recently, Bergquist and Källén (see 1954) have described a metameric distribution of mitoses underlying the early brain differentiation. These authors have revived the controversial issue of the significance of neuromeres. They have found, consistently, in all lower and higher Vertebrate embryos that were investigated, not one but three successive sets of metameric swellings of the brain wall which they call proneuromeres, neuromeres, and transverse bands, respectively. These swellings are the result of local, metamerically spaced centers of proliferative activity. The last set, the transverse bands, are supposed to persist as *"migration areas"* which give rise to waves of migration of neuroblasts toward the periphery. In the mantle, these

strands of cells break up into smaller units which the authors have tried to homologize with specific brain centers. This latter relationship has been contested and it requires further clarification; but irrespective of this point, these studies indicate again a close tie-up of proliferation with subsequent events. If mitotic patterns set the stage for the subsequent differentiation patterns, then an analysis of the factors controlling proliferation would bring us one step closer to the starting point of the sequence of causal relations.

Two types of *cell migration* occur in neurogenesis: the migration of individual cells from the ependymal layer to the mantle which is part of the life history of every neuroblast; and secondary migrations of groups of more or less differentiated neuroblasts within the mantle (further discussion in Hamburger and Levi-Montalcini, 1950). The latter are perhaps more frequent than has been realized; they play a significant role in the formation of the complex structural pattern of the central nervous system, and particularly of the brain. We cannot discuss this topic in detail but must limit ourselves to two illustrations. The dorsal position of the neostriatum which is a motor nucleus in the forebrain has puzzled neurologists. Recent studies carried out by Levi-Montalcini and Williamson (unpublished) have shown that these cells reach their destination by a complicated migration (Fig. 1). In early stages, they are found in a ventrolateral position typical of motor cells; later on, they migrate dorsad and caudad. Similarly, the preganglionic sympathetic nucleus of Terni which is located near the central canal in the thoracic level of the chick spinal cord is not differentiated in situ. Its cells are at first part of the uniform ventrolateral motor column which extends through the length of the spinal cord. At 5½ days, the sympathetic component splits off from the somatic motor component; it moves in a mediodorsal direction and settles medially above the central canal (Levi-Montalcini, 1950). In both instances, the cells which participate in this secondary migration are bipolar neuroblasts whose axons are trailing behind while they migrate.

The phenomenon of secondary migrations confronts us with difficult problems of correlative mechanisms in early neurogenesis. We know that all neuroblasts are endowed with the structural and physiological equipment for ameboid locomotion, but the *directed* movement of neuroblasts presupposes, in addition, some guiding mechanisms or tracks in the underlying substrate ("contact guidance," Weiss) which have not been studied so far.

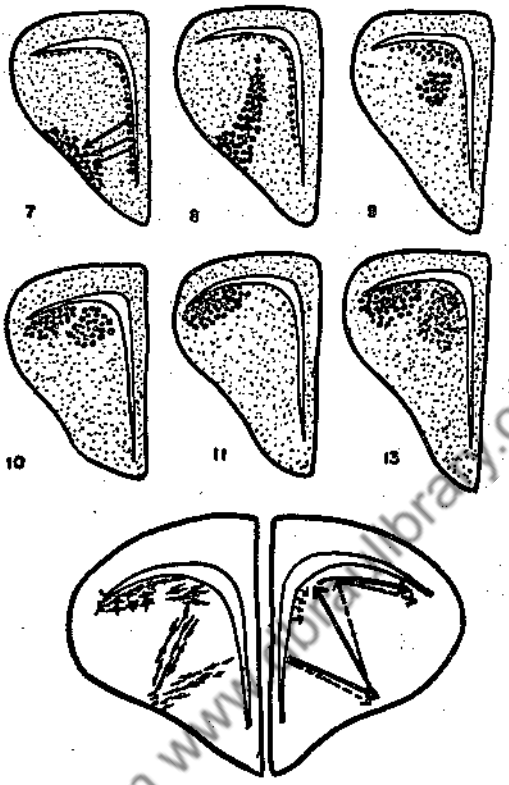


Fig. 1. Migrations in neostriatum (forebrain) of chick embryo. Figures indicate days of incubation. Two major migrations occur in short succession (solid and dotted arrows in lower right figure). The median cell group which appears on day 13 originates locally. (Original, Levi-Montalcini and Williamson, unpublished.)

II. DEVELOPMENTAL CORRELATIONS: INTRODUCTORY REMARKS

Once the basic structural design of an organ is established, the next step of the analysis is directed at the elucidation of the factors which are instrumental in its gradual materialization. In neurogenesis, both intrinsic correlations and interactions with the extrinsic, non-nervous milieu play an important role, particularly in the quantitative aspects of nerve center differentiation and in the maturation and maintenance of nerve cells. It is true that most of these interrelationships are peculiar to the nerve tissue, and the direct outcome of its unique structure. Yet they illustrate at the same time some general principles of organogenesis, and they show that the repertory of determinative mechanisms is by no means exhausted with embryonic inductions which have been in the foreground in the past.

The nerve tissue is unique in the mode of connections of its constituent elements. Through its axon, each neuron establishes a synaptic contact with another neuron or a non-nervous structure; and, with few exceptions, the neuron is also the recipient of a synaptic termination of fibers coming from other neurons. The primary significance of these "double bonds" is of course to be sought in their functional role as transmitters of nerve impulses. However, they are at the same time subservient to other seemingly different physiological functions which are referred to collectively and rather vaguely as "trophic" functions. Both types of connections play an important role in the maintenance of the integrity of the neuron, and in some instances its very survival becomes dependent on its dual synaptic contacts. It is well known from clinical and experimental studies that the severance of the axon from its terminal structure is followed immediately by chromatolysis, and if regeneration is prevented, the regressive changes may continue to the point of complete breakdown of the neuron. In other words, the neuron is dependent on a proper "trophic" equilibrium at its peripheral milieu. We shall refer to this dependency as a "retrograde" or *"peripheral"* trophic relation. On the other hand, regressive changes have been observed in neurons which are denervated, that is, deprived of the synapses which terminate on their cell bodies. These dependencies are known as *"transneuronal"* relations. Some non-nervous tissue, notably muscle tissue, and some sense organs are equally sensitive to denervation. The failure of the Urodele limb to regenerate in the absence of nerves at the stump is another case in point. These findings indicate that neurons release trophic agents at the tips of their axons.

Experimental neuro-embryology has revealed the remarkable fact that both types of trophic interactions, the peripheral and the transneuronal, operate already during the embryonic period. They play a significant role in neuron and nerve center differentiation. The discussion will be focussed on these two points, and a third will be added: the direct effect of chemical growth controlling agents, such as hormones and the "tumor factor" on the pericaryon, without mediation of the axons.

III. DIRECT "PERIPHERAL" EFFECTS ON PRIMARY NERVE CENTERS

When the axons of primary sensory or motor neurons enter their peripheral area of distribution, they immediately establish trophic relationships with their milieu: this is demonstrated by the changes which one observes in the primary sensory or motor centers, following ex-

perimental modifications of the peripheral fields. In a well-known series of investigations, Detwiler analyzed this phenomenon in Urodeles, and his experimental design—the decrease of the peripheral area by extirpation of a limb primordium or its increase by heterotopic limb transplantation—has been followed by many workers. It is essential for an evaluation of these experiments to realize that the peripheral changes are made prior to the outgrowth of nerve fibers and that the latter are not injured. In Urodeles, Detwiler found striking hypo- and hyperplastic effects in the spinal ganglia but none in the motor system (see 1936). Later on, other workers have demonstrated such effects in the motor system of Anurans and Urodeles: they are difficult to detect in the latter, because the motor neurons are not clearly segregated in columns, and the effects do not become noticeable until late larval stages.

A detailed analysis of the repercussions following limb bud extirpation in the chick embryo has revealed that hypo- and hyperplasia are the combined effect of several factors and that different nerve centers respond in a different fashion. The most dramatic response is a rapid disintegration of neuroblasts shortly after their fibers have reached the reduced peripheral area. This has been observed first in the spinal ganglia and recently also in the lateral motor system. One can distinguish in the spinal ganglia two types of neuroblasts: early differentiating ventrolaterally located cells (V-L cells) which send out fibers and grow extensively between 3 and 8 days, and late differentiating smaller cells which are located mediodorsally (M-D cells). After wing bud extirpation at 2½-3 days, the V-L cells in the brachial ganglia undergo a large-scale degeneration which reaches its climax at 5 to 6 days; by the 8th day, all but very few neuroblasts of this category have disappeared (Hamburger and Levi-Montalcini, 1949). A similar, rapid and cataclysmic breakdown was observed recently in the lumbo-sacral motor column after a radical leg bud extirpation. The first degenerating cells were found at 5 days, that is 2½ days after operation; they were abundant between 6 and 7 days (figs. 6, 8, 9), and at 8 days the lateral motor column was practically wiped out. Preliminary cell counts indicate that in normal embryos the lateral motor column is numerically complete as early as at the end of the 5th day, that is shortly before the onset of the degeneration process. Hence, the hypoplasia of over 90% which one observes 3 days later can be accounted for entirely by the breakdown of differentiated neuroblasts. Levi-Montalcini and Levi (1942), who had performed the same experiment, came to the same conclusion on the basis of indirect

evidence, though they had not actually observed disintegrating cells in their silver preparations.

It is remarkable, indeed, that the structural integrity and maintenance of a young neuroblast should depend to such an extent on the physiological conditions to which its axon is exposed at the periphery; a very delicate physiological equilibrium must exist at its growth zone. The situation has its close parallel in the equally rapid reactions of motor neurons of newborn mice to leg amputation. In one particular column of the lumbo-sacral ventral horn, 75% of the motor neurons were found to have disappeared within 5 days, and over 90% after 7 days (Romanes, 1946). However, it should be kept in mind that in this experiment the axon was severed, whereas in the embryo it was not injured at all.

What is the physiological basis for this dependency? We cannot answer this question, but perhaps the behavior of the axons at the periphery can give us some clues. During the critical period, between 5 and 7 days of incubation, the normal leg grows extensively in length and the nerves are spun out at the same rate; they branch and establish preliminary connections with the differentiating myoblasts. This is undoubtedly a period of very active neuro-protein synthesis in the pericaryon. On the operated side, the mixed nerves first grow out in a normal fashion; but instead of forming a typical plexus they join in a thick neuroma-like fiber bundle which extends along the lumbo-sacral segments, parallel to the sympathetic chain (Fig. 2). Silver preparations show the fibers taking tortuous and winding courses. They seem to run back and forth, since the neuroma is considerably thicker than the nerves on the normal side, and they seem to end blindly, since usually few bundles emerge from the neuroma, except for short nerves in the sacral region. The physiological disturbance at the periphery which leads to the breakdown of the neuroblasts is obscure. It is conceivable that the interference with the normal outgrowth and branching of the axon upsets the metabolic equilibrium of the entire cell sufficiently to cause its disintegration. Alternatively, one could imagine that there exists normally a metabolic exchange between the axon and the medium in which it grows, and that in the experimental situation the cell is deprived of some essential metabolic requirement.

In the *brachial cord,* large-scale breakdown of motor neurons has also been observed recently after more radical wing bud operations than had been done before.

We have evidence that *different centers respond differentially* to the reduction of their field of nerve distribution. For instance, the late

differentiating M-D cells in the spinal ganglia (see above, p. 196) do not disappear rapidly subsequent to wing bud extirpation, as do the adjacent V-L cells, but they remain intact at least to the end of incubation and merely show an atrophic condition. The *trochlear nucleus* is not dis-

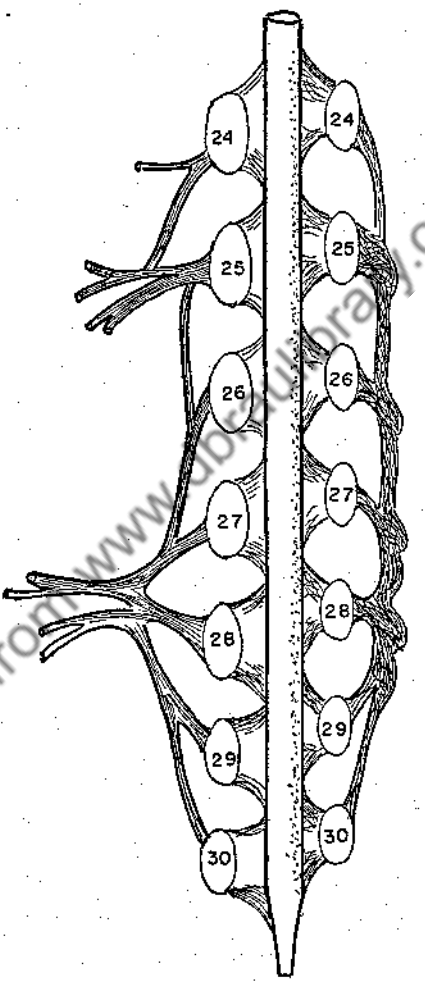


Fig. 2. Seven-day embryo. Neuroma formation after extirpation of left (apparent right) leg bud at 2½ days. (Original.)

turbed in its initial phases of differentiation by the early removal of its peripheral field, the superior oblique muscle. If the optic vesicle including the eye muscle primordia is extirpated at 1½ days of incubation, that is before onset of its differentiation, the trochlear nucleus reaches its full

numerical strength and a normal degree of neuroblast differentiation at 5 days. From then on, there is a gradual cell loss which reaches a 25% level at 8 days and increases gradually up to 86%, near hatching. In contrast to the catastrophic breakdown in the V-L cells of the ganglia and the lumbo-sacral motor centers, we are dealing here apparently with a sporadic disappearance of cells (Dunnebacke, 1953). The situation is remarkably similar in an entirely different system, the *parasympathetic ciliary ganglion* which innervates the ciliary and iris muscles (Amprino, 1943). Following optic vesicle extirpation at 2½-3 days, regressive changes in this structure do not become noticeable until the 8th day, at which stage the ganglion is numerically complete and neuroblast differentiation is in progress. From then on, to the end of incubation, 65-85% of the cells disappear completely, and the remaining cells are atrophic. Likewise, the *sympathetic ganglia* of the brachial level differentiate normally in the absence of the wing, at least up to 8 days, but show a distinct regression at 19 days (Simmler, 1949).

All these experimental data demonstrate conspicuous differences in the responses of different types of neuroblasts to the loss of their peripheral fields. It is possible that they are attributable largely to inherent constitutional differences in the neuroblasts. However, another factor has to be considered: The conditions for axon survival are not strictly comparable in the different types of extirpation experiments. It may make a difference whether the fibers form a neuroma as in the lumbo-sacral region or whether they have an opportunity to spread in adjacent connective tissue or other structures as is the case in some of the other instances.

IV. INDIRECT EFFECTS ON PRIMARY CENTERS: HYPERPLASIA

The extirpation experiments discussed so far have demonstrated a peripheral influence on the maintenance of neuroblasts, mediated by their axons, and a control of the size of the cell population in nerve centers by elimination of cells. This, however, is an incomplete picture. The initial phases of nerve center formation, namely *proliferation* and *initial differentiation* are likewise under peripheral control. Defect experiments are less suitable to demonstrate such effects than experiments of peripheral overloading, by which hyperplastic rather than regressive responses can be elicited. The implantation of supernumerary limbs stimulates an increase of the mitotic activity in the sensory ganglia which are involved in their innervation. This was established for the Urodeles by cell counts (Detwiler, see 1936, a.o.) and for the chick by direct mitotic counts

(Hamburger and Levi-Montalcini, 1949). A slight numerical hyperplasia was also observed in the lateral motor column of Anurans (May, 1933) and of chick embryos (Hamburger, 1939). Similar effects were obtained for the mesencephalic V nucleus in the brain which is unique in that it represents an intracentral sensory center of proprioceptive neurons for jaw muscles. The primordium of the mandibular arch of *Amblystoma punctatum* was replaced by one from the larger *A. tigrinum* embryo resulting in a consistent increase in the cell number of the nucleus (Piatt, 1946).

These effects differ in an essential point from the regressive changes discussed before: they concern cells which have no direct connection with the periphery; hence, they must be indirect. The concept has been developed that stimulative influences spread from the neuroblasts which have sent their fibers to the periphery to adjacent undifferentiated cells; the latter are thus stimulated to increased proliferative activity and differentiation (Barron, 1943, 1946; Hamburger and Keefe, 1944).

V. EFFECTS OF THE TUMOR AGENT

Analysis of embryonic induction was greatly enhanced by the discovery of abnormal inductors (adult tissues, etc.); and similarly, the analysis of growth control in the nervous system was facilitated by the discovery of an atypical growth stimulating agent. It was found that pieces of mouse sarcoma implanted into the coelom of 3- to 4-day chick embryos grow profusely; once established, they are invaded by large numbers of fiber bundles from adjacent spinal and sympathetic ganglia (Figs. 3, 7). The motor system and parasympathetic ganglia are refractory to the agent. The ganglia which participate in the neurotization of the tumor become highly hyperplastic (Bueker, 1941; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1952). The hyperplastic responses exceed by far those obtained by limb transplantations. Sympathetic ganglia were enlarged 2-6 times and spinal ganglia up to 2½ times normal size. The hyperplasia is the result of a combination of factors. It is due in part to an increase in cell number by increased proliferation and in part to cellular hypertrophy; furthermore, it was found that the rate of differentiation and of fiber outgrowth is accelerated. Thus, two new types of response can be added to the repertory of peripheral effects: *control of cell growth and of rate of differentiation.*

The prevertebral ganglionic complexes near the dorsal aorta and in the adrenal gland which are components of the sympathetic system were likewise strongly stimulated by the tumor (Figs. 4, 5). These ganglia

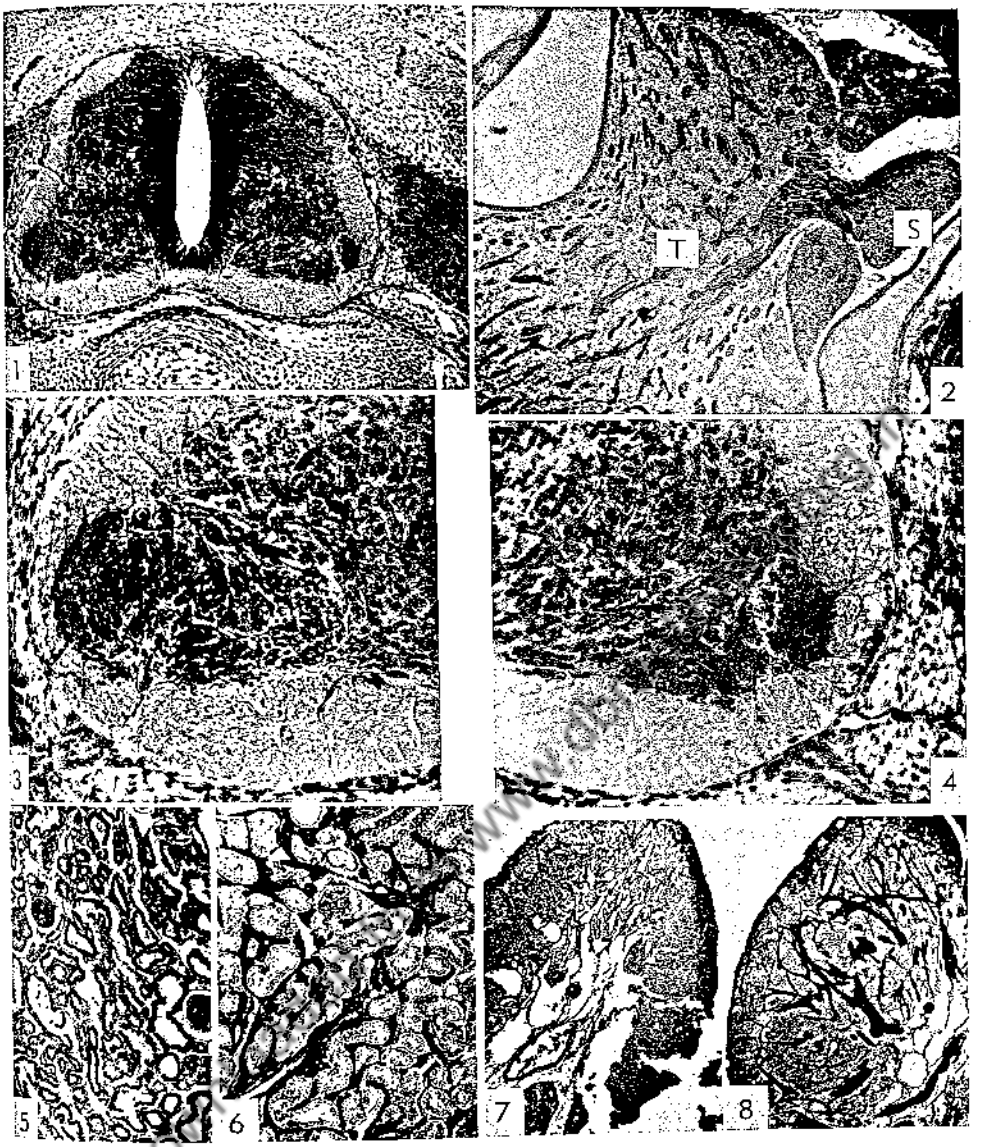


PLATE I. 1. Cross section through lumbo-sacral spinal cord of 7-day chick embryo, following extirpation of left leg bud at 2½ days of incubation. Notice hypoplasia of left (apparent right) lateral motor column. (Hematoxylin.) 2. 11-day chick embryo with neurotized intra-embryonic mouse sarcoma implant (T). A thick fiber bundle from the spinal ganglion (S) enters the tumor. (From Levi-Montalcini and Hamburger, *J. E. Z. 116*, 1951, Fig. 8.) 3. Same as (1). Lateral motor column on normal side. 4. Same as (1). Lateral motor column on operated side. Notice degenerating cells. 5. Mesonephros of a normal 16-day chick embryo. Notice absence of nerve fibers. 6. Visceral nerve fibers in the mesonephros of a 16-day chick embryo with an intra-embryonic tumor. 7. Ovary of a normal 16-day chick embryo. Notice absence of nerve fibers. 8. Visceral nerve fibers in the ovary of a 16-day chick embryo with intra-embryonic tumor. (Figs. 5-8 above from Levi-Montalcini, *Annals N.Y. Acad. Sci. 55*, Figs. 13-16; Figs. 1, 3, and 4 above originals.)

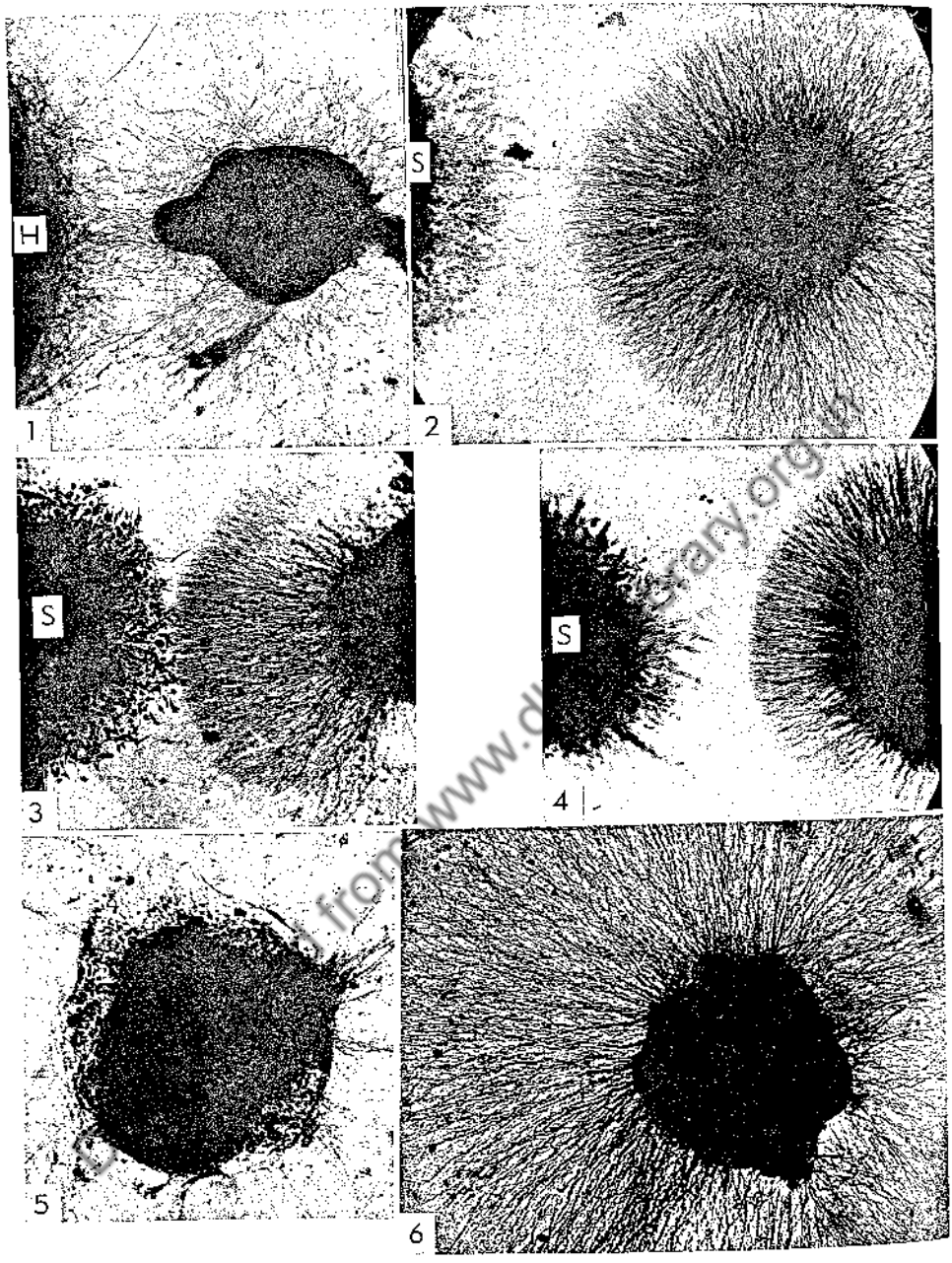


PLATE II. 1. Tissue culture of paravertebral sympathetic ganglion of 13-day chick embryo combined with heart of chick embryo (H) as a control. Cultured for 36 hours. 2. Tissue culture of lumbar spinal ganglion of 7-day embryo combined with sarcoma 37 (S). Cultured for 48 hours. 3. Tissue culture of paravertebral sympathetic ganglion of 13-day chick embryo, combined with sarcoma 37 (S). Cultured for 44 hours. 4. Tissue culture of lumbar spinal ganglion of 7-day chick embryo, combined with sarcoma 37. Cultured for 48 hours. 5. Tissue culture of paravertebral sympathetic ganglion of 10-day embryo. Cultured for 24 hours. Control. 6. Tissue culture of paravertebral sympathetic ganglion, to which was added a drop of "nucleoprotein" fraction from the tumor. Cultured for 24 hours. Compare with (5). (Figs. 1-4 above from Levi-Montalcini, Meyer, and Hamburger, 1954, *Cancer Res. 14*, Figs. 10, 12, 16, and 18; Figs. 5 and 6 above originals.)

which are normally cell clusters of moderate size were occasionally transformed into large, bulky cell strands, with a volume increase of 200 to 400%, and in one case to over 700%. Occasionaly conspicuous ganglionic masses were found embedded in the tumor tissue, particularly when the tumor had reached the level of the adrenal complex. These supernumerary masses have no equivalent in normal embryos; they originate probably by migration from the adrenal complex.

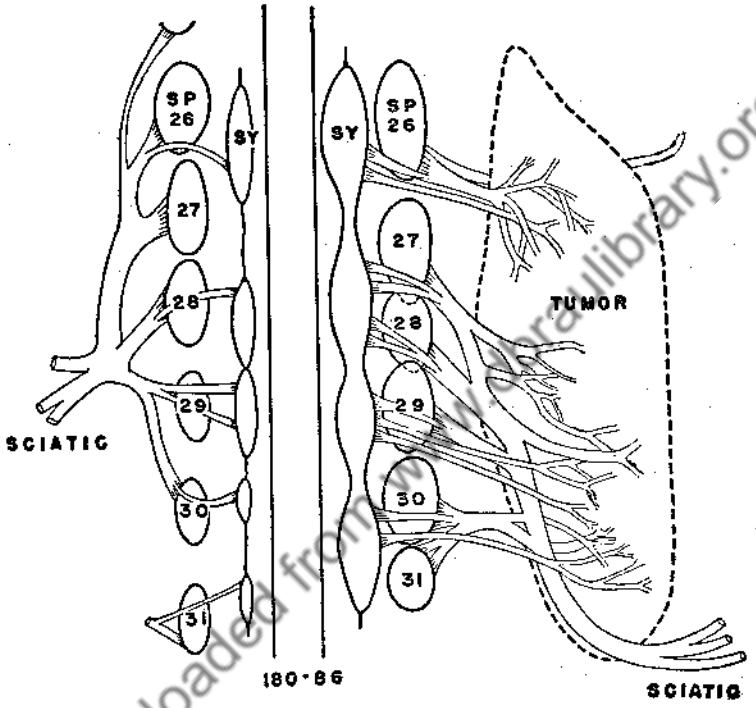


Fig. 3. Neurotization of a mouse tumor (sarcoma 180) in a 15-day chick embryo. Notice hyperplasia of spinal ganglia (SP) and sympathetic ganglia (SY). (From Levi-Montalcini and Hamburger, 1951, Fig. 3.)

This extraordinary overgrowth not only testifies to the strength of the growth promoting activity of the sarcoma but it reveals at the same time remarkable growth potentialities in the sympathetic primordia which remain normally dormant.

In the course of these studies, Dr. Levi-Montalcini noted that some of the hyperplastic para- and prevertebral sympathetic ganglia had no fiber connections with the tumor but, instead, sent their axons into the adjacent mesonephros and other viscera, (Fig. 4B). This observation

led to the idea that the growth promoting agent might be diffusible. To test this hypothesis, tumor pieces were transplanted onto the allantoic membrane of 4-day embryos where they established themselves quickly and grew often to considerable size. The effects of these extra-embryonic tumors on the nervous system of the host were identical with those of the intra-embryonic tumors, and of the same order of magnitude (Fig. 4C). In both instances, the responses were highly selective; they were limited to the sympathetic system, including the pre- and paravertebral ganglia, the superior cervical ganglion and the Remak ganglion, whereas

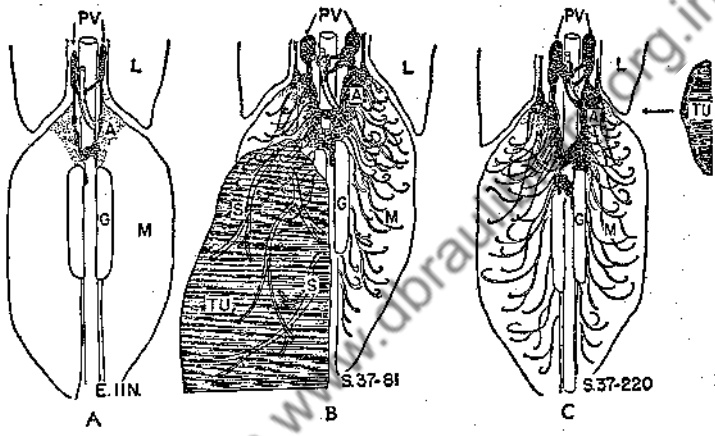


Fig. 4. Diagrams of 11-day chick embryos. A. Normal. B. With intra-embryonic mouse sarcoma 37. C. With mouse sarcoma 37 growing on the allantoic membrane. Notice hyperplasia of prevertebral ganglia (PV) and neurotization of mesonephros (M) in B and C. A, adrenal; G, gonad; L, lung; M, mesonephros; PV, Prevertebral ganglia; S, sensory nerves; Tu, tumor. (From Levi-Montalcini, 1952; Fig. 4.)

the somatic motor and the parasympathetic system (vagus, ciliary ganglion, intramural enteric system) were refractory. These experiments suggest that in all probability the tumor agent is carried in the blood stream (Levi-Montalcini 1952; Levi-Montalcini and Hamburger, 1953).

The extra-embryonic tumors were inaccessible to the nerve fibers; instead, very large and numerous fiber bundles emerging from the hyperplastic ganglia invaded and flooded the adjacent viscera, such as meso- and metanephros, adrenal glands, gonads, thyroid and parathyroid glands. These structures have normally little or no innervation in corresponding stages of development (Fig. 5 and Plate I, 5-8). Synaptic terminations were never found in the hyperneurotized organs. One gets the impression that the fibers are unrestricted in their growth; they seem to take winding, tortuous, recurrent courses in the stroma; they wrap

themselves around blood vessels and kidney tubules, and are entangled in a neuroma-like feltwork which becomes increasingly dense. The most remarkable feature in these cases is the penetration of fibers into the endothelia of veins where they form dense nodules which project into the lumen, occasionally occluding it. Dozens of such nodules of all sizes may be found in one embryo. Altogether, one gets the impression that we are dealing here with a pathological change either in the nerve fiber or in the endothelia or in both.

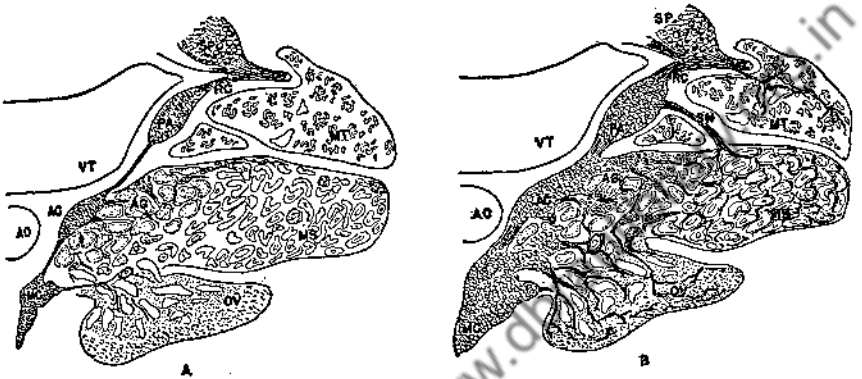


Fig. 5. Diagrammatic cross-section at the level of mesonephros (MS), metanephros (MT), and ovary (OV) to show hyperneurotization. A. Normal 12-day embryo. B. 12-day embryo with extra-embryonic tumor. AC, adrenal complex; AG, adrenal gland; AO, dorsal aorta; MC, mesenteric complex; PA, paravertebral sympathetic ganglion; RC, ramus communicans; SN, supernumerary nerve; SP, spinal ganglion; VT, vertebra. AC and MC are prevertebral sympathetic ganglia. (From Levi-Montalcini and Hamburger, 1953, Fig. 3.)

At this point, it seemed of particular interest to attempt a *chemical characterization* of the tumor agent. Preliminary experiments of implanting frozen-dried tumor or injecting extracts into the embryo were unsuccessful, and Dr. Levi-Montalcini conceived the idea of using the tissue-culture method for this purpose. Individual spinal and sympathetic ganglia from 6- to 10-day embryos are placed on the plasma clot at a distance of 1-2 mm from a small piece of sarcoma. Within 24 hours, a dense "halo" of nerve fibers grows out radially in all directions; in control cultures only a few fibers are formed which usually take irregular winding courses (Levi-Montalcini, Meyer, and Hamburger, 1954; see Plate II, 1-4). This clear-cut and rapid response was then used as a bio-assay for the test of tumor extracts. The biochemical work was done, in collaboration with Dr. Stanley Cohen. Cell-free tumor extracts can be applied by adding the fraction to the medium at the moment of the coagu-

lation of the plasma clot (⅓ plasma, ⅓ synthetic medium, and ⅓ fraction to be tested in a total volume of approximately 0.1 cc). The effects are recorded after 24 hours in terms of 4 grades estimated on the basis of length and density of the fibers. Cell-free homogenates of the tumor duplicated the effect of the growing tumor. Differential centrifugation of homogenates in a 0.25M sucrose solution at *p*H 7.4 eliminated nuclei and mitochondria which are inactive. All activity was retained in the microsome fraction. The active fraction is nondialyzable against water or saline and heat sensitive at 80°C. The agent was purified further by streptomycin sulfate precipitation (0.02M; *p*H 7.2). The precipitate retains all activity; it contains all nucleic acids, as was shown spectroscopically, and some proteins. Hence the agent is associated with a highly polymerized nucleoprotein. Chloroform treatment after removal of the streptomycin gave a highly active supernatant which contains 66% proteins, 27% RNA and 0.2% DNA (Figs. 18, 19). Ashing this fraction completely destroys its activity. Extracts prepared from mouse liver, mouse muscle, and chick embryos were not active (Cohen, Levi-Montalcini, and Hamburger, 1954). The activity was not destroyed when the fraction was preincubated with the following enzymes for 1 hour at 26°C: trypsin, papain, crude pancreatic protease, ribonuclease, desoxyribonuclease, hyaluronidase, lyozymase, steapsin, and amylase. The enzymes were added at a concentration of from ½ to 2% of the dry weight of the fraction (Cohen and Levi-Montalcini, 1955 and unpublished). Subsequent fractionation with ammonium sulfate has yielded an active fraction which contains proteins and only traces of nucleic acids, as shown by absorption spectrophotometry. It possesses 25-50% of the original activity in approximately 1% of the protein content of the tumor, (Cohen and Levi-Montalcini, unpublished). These data, and the finding that the activity is not destroyed by ribonuclease, desoxyribonuclease, dilute alkali, and ultraviolet radiation are taken as evidence against the involvement of nucleic acids in the activity of the tumor agent.

The results of several experiments indicate that we are not dealing with a virus-like, self-reproducing particle. Exposure of the ganglia to the active material for periods up to ½ hour, preceding their explantation into a control medium, did not result in a precocious outgrowth of nerve fibers. Likewise, ganglia which were grown in a medium containing the active agent, for 5 or 24 hours, respectively, and then transferred to a control medium, behaved like control ganglia. Furthermore, ganglia which were removed from a host bearing an intra-embryonic tumor and

explanted into a control medium, did not show an excessive growth (Cohen and Levi-Montalcini, unpublished). These data suggest that the continued presence of the agent is necessary for stimulation of excessive and precocious nerve fiber outgrowth.

To summarize: we are dealing with a specific agent, probably a protein, which stimulates selectively fiber outgrowth in spinal and sympathetic ganglia, but not in parts of the central nervous system, nor in parasympathetic ganglia. So far, it has been found only in mouse sarcomas. It is not contained in neuroblastoma nor in rhabdomyosarcoma C_3H (Bueker and Hilderman, 1953).

VI. NEUROBLASTS AS DIRECT TARGETS OF HUMORAL AGENTS

The discovery of the effects of extra-embryonic tumor necessitates a re-evaluation of other problems, including that concerning the mode of action of the "peripheral" factors. The hyperneurotization of the intra-embryonic tumors and the subsequent hyperplasia of the ganglia could be fitted readily into the old scheme in which the axons mediate the effects between the periphery and the centers from which they originate. But it is difficult to apply this concept to the extra-embryonic tumors which operate at a distance through a humoral agent. One would have to assume that the primary effect would be on the viscera which would somehow become more "receptive" to the ingrowth and support of nerve fibers than they normally are. This possibility cannot be ruled out, but it is more plausible to envisage a direct effect on the ganglia. The agent would enhance directly the growth and differentiation of the neurons and the excessive outgrowth of nerve fibers which would find a natural outlet in the adjacent viscera. It is not even necessary to postulate the breakdown of a protective mechanism against hyperneurotization. Perhaps the embryonic viscera like meso- and metanephros are always accessible to nerve ingrowth, and the sparsity of their normal innervation in early stages may be due to the small amount of fiber material that is normally available.

The tissue-culture experiments, in which ganglia are confronted with tumor pieces at a distance, give support to this idea. The initial fiber outgrowth is not directed toward the tumor, but it occurs simultaneously in all directions, before a bridge is established between the two structures. In this experiment, the ganglion cells are undoubtedly the direct target of the tumor agent. One could argue that the mechanisms *in vitro* and *in vivo* are not necessarily the same. This may be granted, but, nevertheless, the tissue-culture experiment leaves no doubt that neuroblasts *can*

be affected directly by the tumor agent, without mediation of axons. It is conceivable that the intra-embryonic tumors produce hyperplasia of ganglia in a dual fashion, by diffusion to the cell bodies and by supplying the invading fibers with an optimal substrate for growth.

Such a "dual control" is not without precedent. The mesencephalic V nucleus of Amphibians is another example. It was mentioned above that the growth of its cells is controlled by their peripheral field, the proprioceptive sense organs in the jaw muscles. It was observed that cell growth in this nucleus is greatly accelerated shortly before and during metamorphosis (Kollros and McMurray, 1955), suggesting an influence of the thyroid hormone. This assumption was tested by implanting small pellets of crystalline thyroxin adjacent to the anterior wall of the midbrain of frog larvae. It was found that the mesencephalic V neuroblasts in the neighborhood of the implant attained their postmetamorphic size precociously (Kollros, a.o., 1950). A highly selective and differential response to the thyroid hormone was observed by Weiss and Rossetti (1951). These authors studied in a similar fashion the giant Mauthner cells in the hindbrain of Anurans which break down and disappear during metamorphosis, and the adjacent neuroblasts which undergo considerable growth during the same period. Fragments of rat thyroid or agar pieces soaked in thyroid were implanted near the hindbrain of Rana tadpoles. Under the impact of the diffusing hormone, the regressive changes of the Mauthner cells were precipitated ahead of schedule, and at the same time the nuclear volume of the adjacent mantle cells increased up to 83%.

VII. TRANSNEURONAL CORRELATIONS

Nerve cells are not only the generators and transmitters of nerve impulses but also the source of *trophic agents* which are indispensable for the maintenance of other neurons and of some other structures such as muscle and sense organs of the skin. Trophic relations between neurons, which are referred to as *"transneuronal"* effects, can be demonstrated most convincingly in nerve centers which receive fibers from only one source and can therefore be easily denervated. For instance, the cells of the lateral geniculate body in mammals can be completely deafferented by transection of the optic nerve; they have apparently no other afferent synaptic connections. Chromatolysis and cellular atrophy are noticeable in cats and rabbits 2-3 months after operation (Cook, a.o., 1951), and earlier in primates and man. The *superior cervical ganglion* can be isolated by transection of the cervical sympathetic trunk resulting (in the

rabbit) likewise in a cellular atrophy which is detectable at about 40 days after operation (Hamlyn, 1954). Degenerative changes in several thalamic centers following decortication in the opossum were also ascribed, in part, to transneuronal effects (Bodian, 1942).

The problem of trophic "transneuronal" correlations has interesting implications when applied to embryonic stages. What happens to a nerve center which is prevented from ever receiving afferent connections? The most extensive experimental data are available for the embryonic correlations between optic fibers and the optic lobes. The results do not give a uniform picture partly because the reactions seem to be different in different groups, and partly because the data are inadequate. All too often in this and in other neuro-embryological areas, inferences are drawn from observations on one terminal stage, without an analysis of proliferation, differentiation, increase in cell population, and other processes that occur in intervening stages, and without due consideration of details of the normal neurogenetic process.

In Teleost, Amphibian, and chick embryos the hypoplasia of the contralateral optic lobe which follows the unilateral eye vesicle extirpation is very pronounced. Nevertheless one should not overlook the fact that in all instances the general pattern of its differentiation including the typical stratifications proceed in a normal fashion. How does the hypoplasia come about? In the chick embryo, a difference between the affected and the unaffected side cannot be detected until the 13th day. At that stage, proliferation has ceased and the different strata seem to be numerically complete. Hence the subsequent reduction in volume and cell number seems to be due entirely to regressive changes, that is, to cellular atrophy and cell loss (Filogamo, 1950; Bernstein, 1953). This implies that in the chick embryo, the initial phases of proliferation, differentiation, and migration are independent of the inflow of optic fibers. Their trophic role is similar to that in the adult: they are necessary for the *maintenance* of the neuroblasts which in the embryo regress before they can complete their differentiation.

The situation seems to be different in the frog. In this form, mitotic activity in the midbrain ependyma and the increase in the cell population of the superficial sensory strata continue through metamorphosis. Optic vesicle extirpation results in a significant depression of proliferative activity during larval stages, and the numerical hypoplasia which amounts at metamorphosis to over 50% in the superficial strata is attributed entirely to this reduction in the rate of production of tectal cells (Kollros, 1953). The physiological mechanism by which the inflow of

optic fibers at the surface of the optic tectum controls the mitotic activity in the ependymal layer is obscure. The neuroblasts which do reach the outer layers become atrophic and their dendritic processes remain short and undergo less branching than normally (Larsell, 1931).

Most experiments have demonstrated transneuronal effects by the regressive changes that follow the blocking of fiber ingrowth. It is always desirable in such instances to obtain supporting evidence by the demonstration of excessive growth as the result of an increase in trophic activity. Such evidence was supplied by the experiments of Harrison (1929) and Twitty (1932) in which the optic vesicle of the large *Amblystoma tigrinum* was substituted for that of the smaller *A. punctatum*. In those cases in which the larger optic nerve established normal connections with the contralateral optic lobe of the smaller host, the latter showed a noticeable increase in cell number.

Studies on the *cochlear* centers of the chick embryo by Levi-Montalcini (1949) add a new facet to the problem. If the otocyst which includes the primordium of the 8th ganglion is extirpated in 2-day embryos, the acoustico-vestibular centers in the medulla start their differentiation in the absence of the root fibers. It was found that their development proceeds normally up to 11 days at which stage they are numerically complete. Hence, the initial phases of proliferation, differentiation, and migration are unaffected by incoming fibers, as in the optic tectum. From then on, regressive changes occur, but the different centers respond differentially; the nucleus angularis loses 80% of its neurons between 11 and 21 days, the nucleus magnocellularis loses only 32% and a third cochlear center, the nucleus laminaris, as well as the majority of the vestibular centers show no cell loss at all. It was suggested that a quantitative relationship exists between the degree of hypoplasia and the number of afferent synaptic connections which a nerve center receives. The nucleus angularis apparently receives fibers from only one source, the root fibers. Their absence leaves this nucleus completely deafferented. The nucleus magnocellularis receives in addition, other fibers which "protect" its neurons to some degree against damage resulting from the absence of root fibers. A similar correlation has been postulated for the transneuronal effects in the adult.

Transneuronal effects were observed not only after the extirpation of sense organs, but also as indirect effects following changes at the periphery. For instance, the sensory and internuncial systems in the spinal cord undergo hypoplasia after limb bud extirpation in chick embryos (Hamburger, 1934; Bueker, 1947) and in the mammalian fetus

(Barron, 1945, a.o.). However, these effects were not analyzed in detail and they have not revealed new aspects.

In summary: The transneuronal trophic interactions between developing nerve centers are a significant correlative mechanism in neurogenesis. In their complete absence, the nerve centers fail to complete their development and eventually break down.

VIII. CONCLUDING REMARKS

The interneuronal trophic activities which we have discussed are a small facet of a much broader neurophysiological phenomenon. They are akin to the agencies of neural origin which maintain the structural integrity of musculature and sense organs and are necessary for regeneration in amphibians and crustaceans. They may be related to the neurohumoral substances which play a role in impulse transmission and color change, and to the neuro-secretory activities of specialized neurons in the brain and the adrenal medulla. It remains for the future to elucidate the physiological and biochemical nature of this significant aspect of nerve tissue function.

BIBLIOGRAPHY

Amprino, R. 1943. Correlazioni quantitative fra centri nervosi e territori d'innervazione periferica durante lo sviluppo. *Arch. Ital. Anat. Embr. 49,* 1-40.

Barron, D. H. 1943. The early development of the motor cells and columns in the spinal cord of the sheep. *J. Comp. Neur. 78,* 1-27.

Barron, D. H. 1945. The role of the sensory fibers in the differentiation of the spinal cord in sheep. *J. Exp. Zool. 100,* 431-443.

Barron, D. H. 1946. Observations on the early differentiation of the motor neuroblasts in the spinal cord of the chick. *J. Comp. Neur. 85,* 149-169.

Bergquist, H., and B. Källén. 1954. Notes on the early histogenesis and morphogenesis of the central nervous system in vertebrates. *J. Comp. Neur. 100,* 627-660.

Bernstein, P. 1953. The development of the optic tectum in the absence of the optic tract fibers in the chick embryo. Master's Thesis. Washington University. Unpublished.

Bodian, D. 1942. Studies on the diencephalon of the Virginia opossum. *J. Comp. Neur. 77,* 525-575.

Bueker, E. D. 1947. Limb ablation experiments on the embryonic chick and its effect as observed on the mature nervous system. *Anat. Rec. 97,* 157-174.

Bueker, E. D. 1948. Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbo-sacral nervous system. *Anat. Rec. 102,* 369-390.

Bueker, E. D., and H. L. Hilderman. 1953. Growth-stimulating effects of mouse sarcomas 1, 37, and 180 on spinal and sympathetic ganglia of chick embryos as contrasted with effects of other tumors. *Cancer 6,* 397-415.

Coghill, E. G. 1933. Correlated anatomical and physiological studies of the growth of the nervous system of Amphibia. XI. The proliferation of cells in the spinal cord as a factor in the individuation of reflexes of the hind leg of *Amblystoma punctatum,* Cope. *J. Comp. Neur. 57,* 327-347.

Cohen, S., R. Levi-Montalcini, and V. Hamburger. 1954. A nerve growth-stimulating factor isolated from sarcomas 37 and 180. *Proc. Nat. Acad. Sci. 40,* 1014-1018.

Cohen, S., and R. Levi-Montalcini. 1955. Nerve growth-stimulating factor from sarcoma 180. *Feder. Proc. 14,* 194 (Abs.).

Cook, W. J., J. H. Walker, and M. L. Barr. 1951. A cytological study of transneuronal atrophy in the cat and rabbit. *J. Comp. Neur. 94,* 267-291.

Detwiler, S. R. 1936. *Neuroembryology.* Macmillan, New York.

Dunnebacke, T. H. 1953. The effects of the extirpation of the superior oblique muscle on the trochlear nucleus in the chick embryo. *J. Comp. Neur. 98,* 155-178.

Filogamo, G. 1950. Consequenze della demolizione dell'abbozzo dell'occhio sullo sviluppo del lobo ottico nell'embrione di pollo. *Riv. Biol. 42,* 73-80.

Hamburger, V. 1934. The effects of wing bud extirpation on the development of the central nervous system in chick embryos. *J. Exp. Zool. 68,* 449-494.

Hamburger, V. 1939. Motor and sensory hyperplasia following limb bud transplantations in chick embryos. *Physiol. Zool. 12,* 268-284.

Hamburger, V. 1948. The mitotic patterns in the spinal cord of the chick embryo and their relation to histogenetic processes. *J. Comp. Neur. 88,* 221-284.

Hamburger, V. 1952. Development of the nervous system. *Ann. N.Y. Acad. Sci. 55,* 117-132.

Hamburger, V., and E. L. Keefe. 1944. The effects of peripheral factors on the proliferation and differentiation in the spinal cord of chick embryos. *J. Exp. Zool. 96,* 223-242.

Hamburger, V., and R. Levi-Montalcini. 1949. Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. Exp. Zool. 111,* 457-502.

Hamburger, V., and R. Levi-Montalcini. 1950. Some Aspects of Neuroembryology. In *Genetic Neurology,* edited by Weiss. Pp. 128-160. University of Chicago Press, Chicago.

Hamlyn, L. H. 1954. Effect of preganglionic section on the neurons of the superior cervical ganglion in rabbits. *J. Anat. 88,* 184-191.

Harrison, R. G. 1907. Observations on the living developing nerve fiber. *Anat. Rec. 1,* 116.

Harrison, R. G. 1929. Correlation in the development and growth of the eye studied by means of heteroplastic transplantation. *Arch. f. Entw.-mech. 120,* 1-55.

Kollros, J. J. 1953. The development of the optic lobes in the frog. I. The effects of unilateral enucleation in embryonic stages. *J. Exp. Zool. 123,* 153-188.

Kollros, J. J., V. Pepernik, R. Hill, and J. C. Kaltenbach. 1950. The growth of mesencephalic V nucleus cells as a metamorphic event in Anurans. *Anat. Rec. 108,* 565 (Abs.).

Kollros, J. J., and V. M. McMurray. 1955. The mesencephalic V nucleus in Anurans. I. Normal development in *Rana pipiens. J. Comp. Neur. 102,* 47-64.

Larsell, O. 1931. The effect of experimental excision of one eye on the development of the optic lobe and opticus layer in larvae of the tree frog (*Hyla regilla*). II. The effect on cell size and differentiation of cell. *J. Exp. Zool. 58,* 1-20.

Levi-Montalcini, R. 1949. The development of the acoustico-vestibular centers in the chick embryo in the absence of the afferent root fibers and of descending fiber tracts. *J. Comp. Neur. 91,* 209-242.

Levi-Montalcini, R. 1952. Effects of mouse tumor transplantation on the nervous system. *Ann. N.Y. Acad. Sci. 55,* 330-343.

Levi-Montalcini, R., and G. Levi. 1942. Les conséquences de la destruction d'un territoire d'innervation périphérique sur le développement des centres nerveux correspondants dans l'embryon de Poulet. *Arch. de Biol. 53,* 537-545.

Levi-Montalcini, R., and V. Hamburger. 1951. Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool. 116,* 321-362.

Levi-Montalcini, R., and V. Hamburger. 1953. A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. *J. Exp. Zool. 123,* 233-288.

Levi-Montalcini, R., H. Meyer, and V. Hamburger, 1954. *In vitro* experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer Research 14,* 49-57.

May, R. M. 1933. Réactions neurogéniques de la moelle à la greffe en surnombre, ou à l'ablation d'une ébauche de patte postérieure chez l'embryon de l'anoure, *Discoglossus pictus* Otth. *Bull. Biol. 67,* 327-349.

Piatt, J. 1946. The influence of the peripheral field on the development of the mesencephalic V nucleus in Amblystoma. *J. Exp. Zool. 102,* 109-142.

Romanes, G. J. 1946. Motor localization and the effects of nerve injury on the ventral horn cells of the spinal cord. *J. Anat. 80,* 117-131.

Simmler, G. M. 1949. The effects of wing bud extirpation on the brachial sympathetic ganglia of the chick embryo. *J. Exp. Zool. 110,* 247-258.

Sperry, R. W. 1951. Regulative factors in the orderly growth of neural circuits. *Growth Symp. 10,* 63-87.

Twitty, V. C. 1932. Influence of the eye on the growth of its associated structures, studied by means of heteroplastic transplantation. *J. Exp. Zool. 61,* 333-374.

Weiss, P. 1941. Nerve patterns: The mechanics of nerve growth. 3rd Growth Symp., *Growth 5,* 163-203.

Weiss, P. 1955. Special Vertebrate Organogenesis; Chapter 1, Nervous System. In *Analysis of Development*, edited by Willier, Weiss, and Hamburger. Pp. 346-401. Saunders, Philadelphia.

Weiss, P., and F. Rossetti. 1951. Growth responses of opposite sign among different neuron types exposed to thyroid hormone. *Proc. Nat. Acad. Sci. 37*, 540-556.

X. STRUCTURAL STUDIES OF THE FORMATION OF THE MYELIN SHEATH IN PERIPHERAL NERVE FIBERS

BY BETTY BEN GEREN [1]

AN UNDERSTANDING of the exact anatomical relationships of the axon, myelin sheath, and Schwann cell has awaited examination of these structures at resolutions higher than the light microscope permits. It is the purpose of this paper to describe studies of these cellular structures carried out with the electron microscope.

Investigation of nerve degeneration after axon section has shown that myelin disappears in the peripheral stump, and that the Schwann cells remain in "tubes." Regenerating axons enter the Schwann cell tubes and acquire myelin sheaths. In the absence of contact between a peripheral axon and Schwann cells, myelin is not formed; in the absence of continuity of the axon with its cell body, the myelin sheath is not maintained. Thus, the myelin sheath is under dual control.

The study of the structure of the myelin sheath at the colloidal and the molecular levels has been pursued for many years. W. J. Schmidt (1937) deduced from polarization optical data that the lipid molecules in the myelin sheath are oriented radially and the protein molecules tangentially. The fundamental radial repeating units of 171Å for Amphibian nerve myelin and of 186Å for mammalian nerve myelin were determined in small angle X-ray diffraction studies by Schmitt et al. (1935, 1941). This large a unit was not found in total lipid extracts of nerve or in purified dried lipids. For this reason, the fundamental radial repeating unit of the myelin sheath was assumed to contain a protein component in addition to the lipids. The interpretation of the X-ray data by the latter group of investigators was based on further measurements of myelin diffractions after drying or after lipid extraction, and on studies of diffraction patterns of purified nerve lipids (phospholipids, cerebrosides and cholesterol); their models included two double layers of mixed lipids (130Å),

[1] The Children's Cancer Research Foundation and the Pathology Department, Harvard Medical School, Boston; and the Biology Department, Massachusetts Institute of Technology, Cambridge. This investigation was supported in part by a research grant (B190) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service, and in part by a grant from the United Cerebral Palsy Association, Inc.

water (about 25Å), and protein (about 20-30Å) in the radial repeating unit. Essentially similar conclusions have subsequently been reached by Finean (1953a).

Fernández-Morán (1950) was the first to observe a radial repeating unit in electron micrographs of thin sections of osmium fixed myelin. The alternation of concentric electron dense and less dense bands in the myelin sheath of osmium fixed nerve fibers (Plate II, 4) has now been observed by many investigators in a wide variety of forms (frog, chameleon, chick, rat, mouse, man).

The highest resolution electron micrograph of myelin that has been published thus far is that of Sjöstrand (1953) who found the repeating period of myelin to be about 120Å; the dense lines were about 30Å thick (close to the limit of resolution in the micrographs) and the less dense lines were about 90Å thick.

Our own investigations have been concerned primarily with the mode of development of the concentric layers of the myelin sheath. Geren and Raskind (1953) published the results of electron microscope studies of cross sections of embryonic chick sciatic fibers. These showed that the older the embryo the greater the number of layers (repeating units of alternate dense and less dense bands) in the myelin sheaths of a majority of fibers. This demonstrated that the process of myelin formation occurs by the orderly addition of concentric layers.

Further study (Geren, 1954) of the embryonic chick sciatic fibers (12-18 days' incubation) revealed that the myelin sheath is formed by a process of infolding of the Schwann cell surface initiated at the time the Schwann cells envelop the outgrowing axons. Fig. 1 summarizes the process illustrated in the electron micrographs of the chick material previously published. These results have now been confirmed by the author in studies of thin transverse sections of sciatic nerves of 7- and 8-day-old mice, as illustrated herein. Plate I, 1 shows the first stage of myelination, that of apposition of the Schwann cell and axon surfaces. In 2, the axon is enveloped by the Schwann cell whose infolded surface is continuous through the cytoplasm as a dense-edged membrane. In 3, the infolded Schwann cell surface is seen as a spiral, continuous (except for small artifacts due to sectioning) from its position at the axon surface to the surface of the Schwann cell. Plate II, 4, includes a region at the edge of a myelinated fiber. The myelin is now a compact layering of dense and less dense bands and one can observe that the dense-edged membrane formed by the infolding Schwann cell surface joins the compact layering by fusion of one of its dense edges with the outermost dense line of the

compact myelin. (Robertson (1955) observed the connections of the infolded Schwann cell surface with the compact myelin in sections of adult chameleon nerve fibers. He showed that the dense lines of the compact myelin sheath are formed by the apposition of the dense edges of ad-

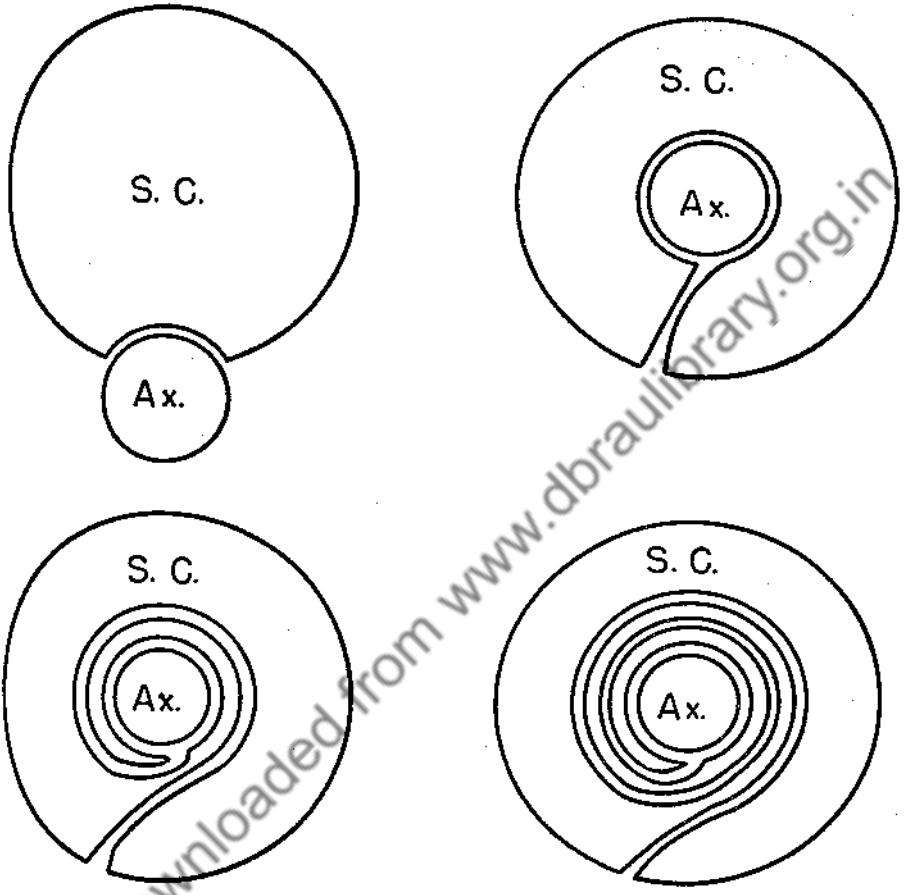


Fig. 1. Diagrammatic representation of the early stages of myelin formation. Ax., axon; S.C., Schwann cell.

jacent layers of the spiral.) The dense lines fuse to form a single dense line of the same apparent thickness as each of the fusing dense lines.

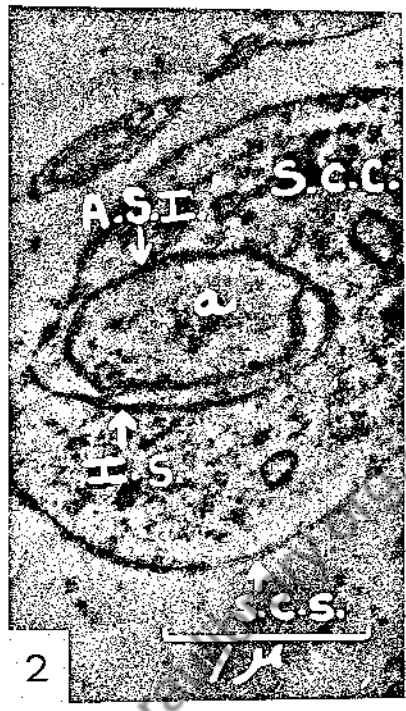
I. INTERPRETATION OF MYELIN SHEATH FINE STRUCTURE

Finean (1954) has discussed the difficulties of formulating an exact molecular interpretation of the alternate dense and less dense bands seen in electron micrographs of the myelin sheath. He made X-ray patterns of

a fixed and embedded nerve fiber from which Sjöstrand had prepared sections for electron microscopy. Finean's X-ray patterns from this fiber showed a fundamental repeating unit of 148Å with a second order diffraction at 74Å. Sjöstrand's measurement of 119Å in the electron micrographs is clearly larger than half the X-ray period and smaller than the full unit spacing. Finean considered many possible reasons for the disparity in the two types of data. Since the data obtained both by X-ray diffraction and by electron microscopy are dependent on electron density distributions within the specimen, one might expect to be able to correlate results from each method. This has not, to date, been possible in terms of a quantitative analysis for myelin sheath fine structure.

Each radially repeating unit of the myelin sheath as seen in electron micrographs is derived from the dense-edged membrane formed by the infolded Schwann cell surface. In compact myelin this spacing would be measured from the center of one dense band to the center of the next dense band. The dense-edged membrane is a true double membrane since it literally represents the apposition of *two* infolded surfaces of the same Schwann cell. The electron microscope evidence indicates that the Schwann cell surface represents the true radial repeating unit of the myelin sheath.

The dense line at the outer border of the Schwann cell may be considered to have an inner surface (on the cytoplasmic side) and an outer surface (on the extracellular side). It can be noted (e.g. on micrograph 4, Plate II) that it is along the inner surface that the dense lines of the spiraled double membrane fuse to form the compact myelin layering, but that the outer surfaces of the dense lines never approximate. We feel that this is evidence that the structural domain of the cell surface extends beyond the dense line visualized at the cell surface in electron micrographs, and that the less dense band in the myelin repeating unit cannot be considered simply as a "space" (i.e., a channel filled only with extracellular salt solution). The width of the central less dense band in the dense-edged membrane formed by the infolded Schwann cell surface varies considerably (100-300Å) and is generally much wider than the average dimension of the less dense bands in the compact myelin (90Å). This decrease in thickness of the central less dense zone may be due either to a loss of fluid or of structural components in the less dense band, or to closer packing in the radial direction of the molecules in the less dense zone.



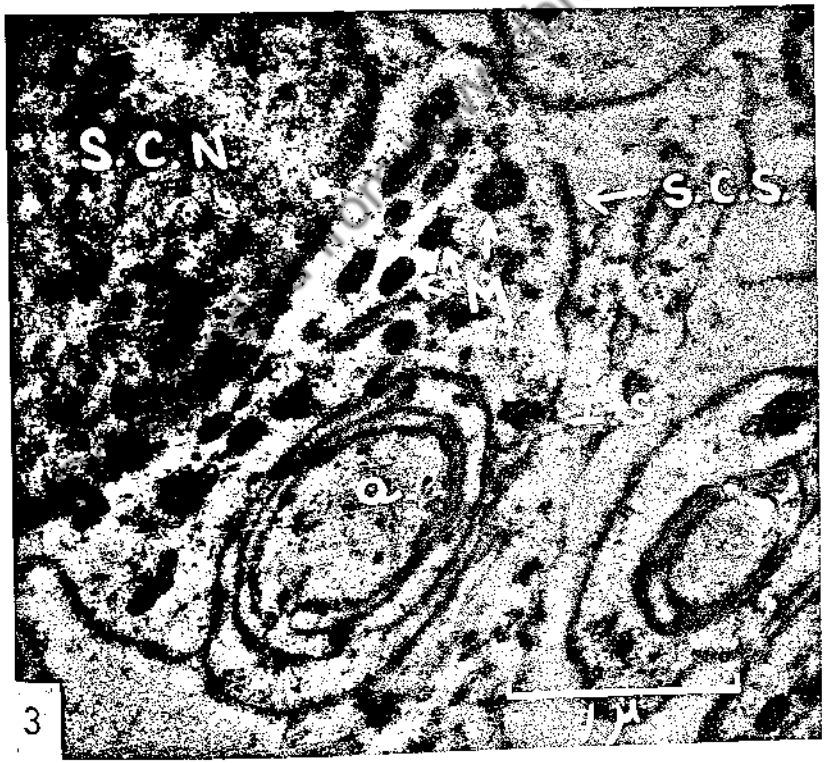
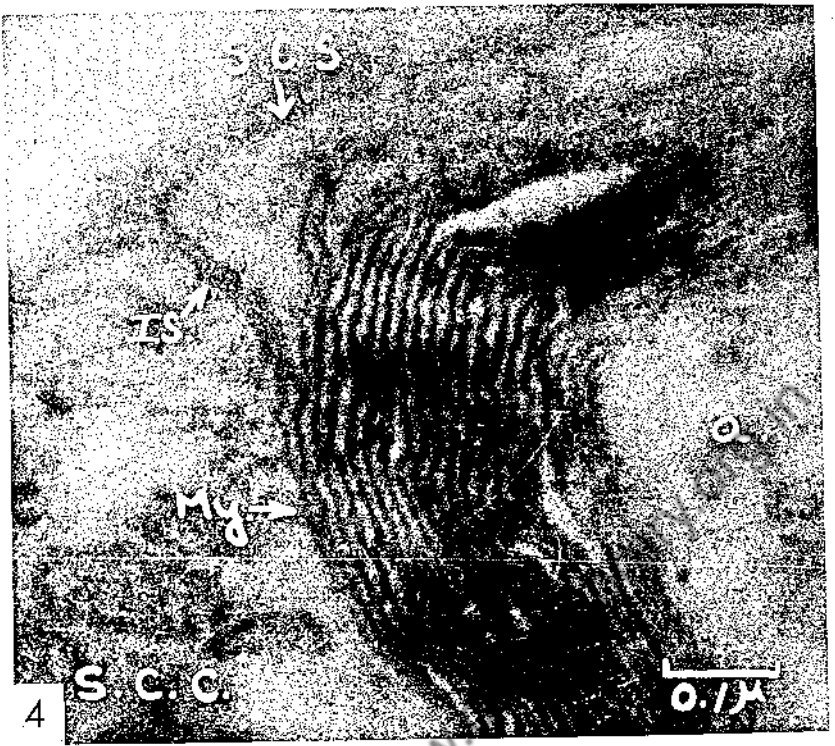
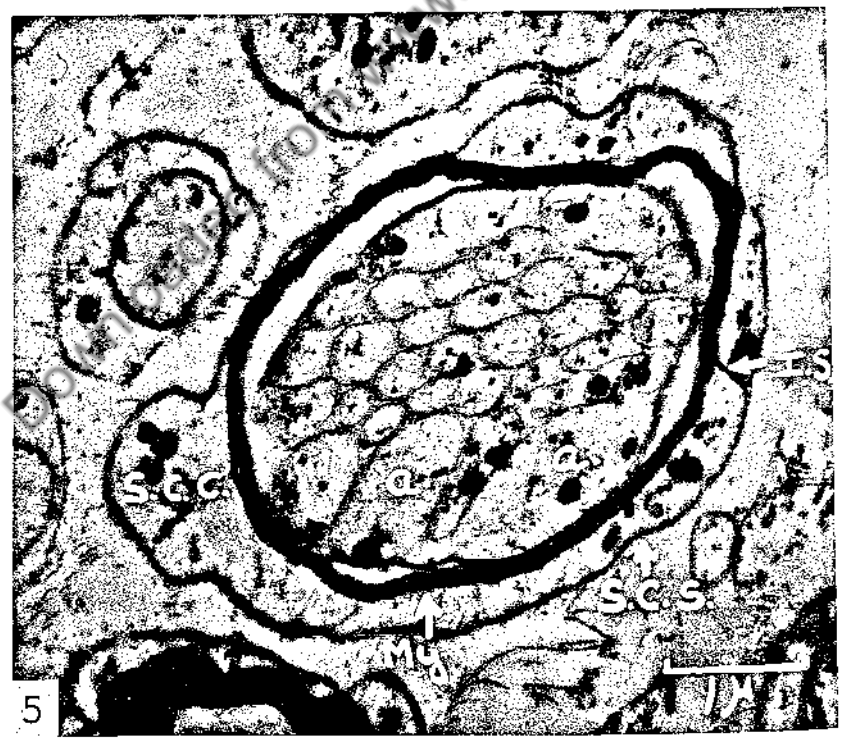


PLATE I. 1-3 (and Plate II, 4 and 5). Electron micrographs of transverse sections through the sciatic nerves of 1- and 8-day-old mice. Material fixed in 1% osmium tetroxide, buffered with veronal acetate at about pH 7.4. 1. Two axons at the surface of a Schwann cell. 2. Axon enveloped by a Schwann cell whose surface is infolded as a dense-edged membrane. 3. Spiral wrapping of infolded Schwann cell surface. a, axoplasm; A.S.I., axon-Schwann cell interface; I.S., infolded Schwann cell surface; M, mitochondria; My, compact myelin; S.C.N., Schwann cell nucleus; S.C.C., Schwann cell cytoplasm; S.C.S., Schwann cell surface.



4



5

PLATE II. 4. Edge of Schwann cell showing infolded surface fusing with outer layer of compact myelin. 5. Compact myelin around 30 axons. Note arrow (I.S.) indicating infolding of Schwann cell surface. (For further explanation, see Plate I.)

II. CELLULAR RELATIONSHIPS

The spiral course of the infolded Schwann cell surface suggests a relative rotation of the axon and/or Schwann cell. Plate II, 5, shows a single myelin sheath wrapped around 30 axons. It is unlikely that all these axons are branches of a single fiber, or that the axonal rotations of 30 different fibers could be coordinated so as to wrap a single myelin sheath about all of them. Such an observation (we have seen this only three times in several hundred sections) suggests that the Schwann cell rotates relative to the axon, thus forming its unfolded cell surface into a spiral double membrane around the axon or group of axons. Clockwise and counterclockwise spirals have been observed in the same electron micrograph.

The mode of formation of myelin by wrapping the infolded Schwann cell surface is consistent with the fact that there is only one Schwann cell per internode in the great majority of peripheral nerve fibers. The clefts of Schmidt-Lantermann in the myelin are not well understood; the present studies on myelogenesis show these clefts to be regions of disorder in the myelin sheath, and studies of longitudinal sections of myelinating fibers may clarify their manner of origin.

We have observed that the myelin around many of the small axons in young animals is of the compact type (i.e., the consecutive layers of the spiraled dense-edged membrane fuse very early). The mechanism that allows for radial growth of the axon must involve some way of expanding this compact myelin sheath.

The formation of myelin in the central nervous system is currently under study. So far, in the white matter of the spinal cord, we have not been able to observe cells analogous to the Schwann cells as we have described them for peripheral fibers, although the concentric layers of the myelin sheath are readily observed. However, in various pathological states, demyelination is frequently limited to areas in either the central or the peripheral nervous system. This may indicate that there is a fundamental biological difference between the myelin sheaths of central fibers and those of peripheral fibers.

III. FUNCTIONAL ROLES OF THE SCHWANN CELL AND THE AXON

During the period of development in which myelin formation is proceeding rapidly, the rate of incorporation of lipid (and protein) into the Schwann cell surface must be high. Although very little is known

about this process from a biochemical point of view, it seems clear that the metabolic activity of the young Schwann cells must be high. The infolding of the Schwann cell surface in Robertson's sections of adult chameleon fibers indicates in the mature nerve the preservation of the embryonic anatomical relationships between axon, myelin, and Schwann cell, and might suggest continuous synthesis of myelin by the Schwann cell.

In addition to the metabolic activity of the Schwann cell during myelin synthesis, the functional roles of this cell in the embryo, as in nerve regeneration, include its selective adhesion to axon surfaces, generally. However, it is clear that there are differences among nerve fibers in their interactions with Schwann cells. This is indicated by the fact that there are fibers with thick myelin sheaths, fibers with thin myelin sheaths, and as Gasser (1955) has shown, fibers imbedded in Schwann cells, but with no myelin sheaths. The axon functions in some way, therefore, as a controlling factor in myelin synthesis as well as in myelin maintenance. Since the axon and the Schwann cell are in surface-to-surface contact only, the factors responsible for axon control of the Schwann cell must either be at the axon surface or be transmitted through the axon-Schwann cell interface.

IV. THE AXON-SCHWANN CELL INTERFACE

The axon-Schwann cell interface (rather inadvisedly called axolemma by Geren in 1954) is a dense-edged double membrane of origin different from the layers of the myelin sheath. The dense line on the axon side of this interface is contributed by the axon surface, whereas the outer dense line of this layer is contributed by the Schwann cell, the central less dense band representing contributions from each type of cell surface. We shall refer to this type of double membrane, derived from the surfaces of two different cell types, as a "complex" double membrane and to double membranes formed by the infoldings of a single cell surface, as "simple" double membranes. It is the complex double membrane of the axon-Schwann cell interface that is constantly observed in all types of peripheral nerve fibers even though the Schwann cell surface, as in the case of the "C" fibers (Gasser, 1955), is not infolded to form a many-layered myelin sheath, or, as in the squid giant fiber (Geren and Schmitt, 1954), is more complexly infolded to form a system of intracytoplasmic double membranes. The complex membrane at the axon-Schwann cell interface may be concerned

in functional roles of major importance: (1) it is a constant anatomical feature of all the types of peripheral nerve fibers studied with the electron microscope, and, as such, may represent a very important component of the excitable mechanism; (2) it is within the domain of this complex double membrane that the axon mediates its influence on the Schwann cell concerning myelin synthesis and myelin maintenance; and (3) it is the site of numerous evaginations into the axoplasm in certain lobster and squid nerve fibers (Geren and Schmitt, 1954). Its continuity (within limits of resolution) with the surfaces of intra-axonal mitochondria has been observed, and its role in relation to mitochondrial activity has been suggested (Geren and Schmitt, 1954).

V. THE MYELIN SHEATH CONSIDERED AS A CELL SURFACE

The concept developed herein regards the myelin sheath as a rolled-up Schwann cell surface. This view seems to be satisfactorily supported by experimental evidence. It would appear, therefore, that a detailed analysis of myelin sheath structure and composition might throw important light on the structure and composition of the cell surface membrane. This assumes, of course, that in the process of infolding and in the subsequent close packing of the layered structure with extravasation of fluid and soluble materials from between the spirally wrapped layers, the essential structure and properties of the surface membrane are not significantly altered.

BIBLIOGRAPHY

Fernández-Morán, H. 1950. Sheath and axon structures in the internode portion of vertebrate myelinated nerve fibers. *Exp. Cell Res. 1,* 309-340.

Finean, J. B. 1953a. Further observations on the structure of myelin. *Exp. Cell Res. 5,* 202-215.

Finean, J. B. 1954. The effects of osmium tetroxide fixation on the structure of myelin in sciatic nerve. *Exp. Cell Res. 6,* 283-292.

Gasser, H. S. 1955. Properties of dorsal root unmedullated fibers on the two sides of the ganglion. *J. Gen. Physiol. 38,* 709-728.

Geren, B. B. 1954. The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. *Exp. Cell Res. 7,* 558-562.

Geren, B. B., and J. Raskind. 1953. Development of the fine structure of the myelin sheath in sciatic nerves of chick embryos. *Proc. Nat. Acad. Sci. 39,* 880-884.

Geren, B. B., and F. O. Schmitt. 1954. The structure of the Schwann cell and its relation to the axon in certain invertebrate nerve fibers. *Proc. Nat Acad. Sci. 40,* 863-870.

Robertson, J. D. 1955. The ultrastructure of adult vertebrate peripheral myelinated fibers in relation to myelinogenesis. *J. Biophys. and Biochem. Cyt. 1*, 271-278.

Schmidt, W. J. 1937. Über die Formdoppelbrechung des osmierten Markscheide des Nerven. *Z. wiss. Mikroskopie 54*, 159-166.

Schmitt, F. O., R. S. Bear, and G. L. Clark. 1935. X-ray diffraction studies on nerve. *Radiology 25*, 131-151.

Schmitt, F. O., R.S. Bear, and K. J. Palmer. 1941. X-ray diffraction studies on the structure of the nerve myelin sheath. *J. Cell. Comp. Physiol. 18*, 31-42.

Sjöstrand, F. S. 1953. The lamellated structure of the nerve myelin sheath as revealed by high resolution electron microscopy. *Experientia 9*, 68-69.

XI. ACQUIRED TOLERANCE OF FOREIGN CELLS

BY R. E. BILLINGHAM [1]

I. INTRODUCTION

It is now a commonplace of both experimental and surgical experience that most tissues of the body do not long survive their transplantation between randomly chosen individuals of the same mammalian species. For example, skin homografts, which have been studied with particular care because of the ease with which they can be handled experimentally, heal in at first and enter a phase of epithelial proliferation as do skin autografts, i.e. grafts of the recipient's *own* skin. Sooner or later, however, they become inflamed, their blood vessels undergo disruption, and they are reduced to discolored scabs which slough off. The destruction of skin homografts is usually complete within 8-15 days and is the outcome of an actively acquired immunity response on the part of the host. At least in general outline this response is similar to the more familiar defense mechanism whereby an animal reacts to infection with heterologous antigens such as bacterial cells.

The main factors which determine the time of onset of a host's reaction against grafts of homologous skin are (a) the amount which is grafted: the higher the dosage, the shorter the survival time; and (b) the antigenic and therefore the genetic relationship between donor and recipient: the smaller the disparity the longer the survival time of the grafts. This second factor is of much greater moment than the first. Once an animal has reacted against homografts from a donor, its resistance is heightened—it has become immunized or sensitized—so that it reacts against subsequent grafts from the same donor (or from another donor of the same genetic constitution) much more rapidly. The blood vessels of "second set" homografts undergo premature disruption, and there is complete suppression of epithelial proliferation. These are diagnostic signs of an "immune" state.

The subject matter of this paper is the phenomenon of acquired tolerance, which may be regarded as exactly the opposite of actively acquired immunity, and is brought into being by the exposure of mammals or birds to foreign cells during their embryonic life.

[1] Department of Zoology, University College, London.

II. THE BEHAVIOR OF SKIN HOMOGRAFTS EXCHANGED BETWEEN DIZYGOTIC CATTLE TWINS

A few years ago my colleagues and I (Anderson, Billingham, Lampkin, and Medawar, 1951; Billingham, Lampkin, Medawar, and Williams, 1952), not knowing at the time of Owen's work, carried out experiments to find out whether the interchange of skin homografts between the members of pairs of young cattle twins would provide a reliable and objective method for distinguishing between those of monozygotic and those of dizygotic origin. The principle is simple enough: since homografts do not long survive transplantation between individuals of ordinary genetic diversity, grafts between two-egg twins should soon break down, for their genetic relationship is no closer than that of full siblings of separate birth; grafts between one-egg twins, on the other hand, should survive indefinitely, for in effect they are autografts.

Misgivings that graft dosage might be a serious technical obstacle proved to be ill-founded, for the amount of skin which could be grafted to cattle was relatively minute compared with that which can be grafted to laboratory rodents. Preliminary trials showed that the violence of the homograft reaction in cattle is such that grafts (in most cases groups of 4-6 small, full-thickness discs of skin about 1 cm in diameter) exchanged between unrelated animals of the same breed were completely destroyed within 15 days. The strength of the reaction was found to be no less severe in the case of grafts exchanged between full siblings of separate birth, or between a dam and her offspring. As anticipated, the grafts exchanged between twins classified as monozygotic by the usual method of phenotypic appraisal provoked no reactions. However, to our surprise the great majority of the twins of similar sex classified as dizygotic were also tolerant of their grafts. To exclude the rather remote possibility that this was the outcome of mistaken diagnosis—that the animals were in fact monozygotic—grafts were exchanged between twins of dissimilar sex, and therefore of unquestionable dizygosity, with precisely the same result.

Of a considerable number of dizygotic twins tested, all displayed a high degree of tolerance to homografts from their respective partners, for no homograft lived for less than 70 days. The majority of the animals were completely tolerant of their homografts within the time limits of the experiments, which in most cases considerably exceeded

100 days. In some animals the grafts underwent low-grade reactions, revealed by a transient inflamed condition with minor imperfections of the epidermis, from which they subsequently recovered.

In those animals whose grafts did eventually break down the reactions were greatly delayed in onset and were of chronic, low-grade intensity, differing conspicuously from those seen in the controls. Moreover, these dizygotic twins were by no means necessarily symmetrical in their responses; grafts transplanted from one twin to the other might be tolerated throughout the observation period, despite the fact that the grafts reciprocally transplanted were eventually destroyed. The tolerance that these dizygotic twins exhibited to grafts of each other's skin was completely specific, for they reacted with normal vigor against homografts from their dam, from brothers and sisters of separate birth, or from unrelated animals.

III. THE CAUSE OF THE ANOMALOUS TOLERANCE OF DIZYGOTIC TWINS TO HOMOGRAFTS OF EACH OTHER'S SKIN

Bovine twins are normally synchorial and, as Lillie (1916, 1917) demonstrated, they actually establish a vascular anastomosis during fetal life so that a constant interchange of blood takes place between them. Lillie's endocrinological interpretation of the "freemartin" condition—the sterility and reproductive abnormality of female calves twin-born with males—turned upon this vascular connexion during fetal life. The normal fertility of about 8% (Swett, Matthews, and Graves, 1940) of females in dissimilar twin pairs was ascribed to the fact that no vascular connexion had been established between the embryos.

It has also been discovered that the majority of dizygotic twins have identical red cell antigens (Owen, 1945), although upwards of 40 red cell antigens are known in cattle (Stormont and Cumley, 1943) and uniformity of red cell antigens between siblings other than twins is rare. Evidence was produced by Owen that at birth and long afterwards the blood of dizygotic twin cattle may contain a mixture of red cells of two antigenically different types. Since red cells are short-lived and do not reproduce, Owen inferred that red cell precursors are exchanged between the twins *in utero*, through the vascular anastomosis that unites them, and survive thereafter as cellular homografts. Therefore, at birth the dizygotic cattle twin is already a chimera. The finding

that skin homografts interchanged between dizygotic twins after birth are in a high degree, though not always permanently, mutually acceptable, is fully consistent with Owen's hypothesis.

It has been found that the proportion of dizygotic twin cattle that showed complete—as opposed to partial—tolerance to grafts from their respective twins corresponds fairly closely with the proportion of females in twin pairs of dissimilar sex that reveal some degree of sexual abnormality. This may be taken as evidence that, at least in cattle, homograft tolerance and female sterility share at least one necessary causal condition in common: the confluence of fetal circulations. Stone, Stormont, and Irwin (1952) have shown that in twin cattle of dissimilar sex there is a complete correlation between infertility and red cell chimerism; cows that are not chimeras are not freemartins.

From the above facts it was inferred that the state of tolerance demonstrable in dizygotic cattle twins was the outcome of their exposure to foreign homologous cells early in embryonic life; in some way this appeared to have rendered them specifically incapable of reacting immunologically against those cells in later life after their faculty for immunological response had developed (see Burnet and Fenner, 1949). The fact that the tolerance resulting from the interchange of red cell precursors, and doubtless other types of cell too, should extend to skin homografts is not surprising: a wide overlap exists between the antigens of different types of tissue cells in the body.

IV. THE EXPERIMENTAL INDUCTION OF TOLERANCE

To test the hypothesis that was advanced to account for the tolerance of dizygotic cattle twins, experiments have subsequently been carried out in which mice, rabbits, rats, and chickens were exposed to living homologous tissue cells early in their embryonic life (Billingham, Brent, and Medawar, 1953, 1955, and 1956). The principle of the experimental procedure was as follows: the embryos were inoculated *in utero* (or *in ovo*) as early as possible with the immunologically "foreign" cells; then, after birth and the attainment of a fully matured immunological response mechanism, the animals were challenged with skin homografts from the original donor of the fetal inoculum or, in the case of the mice, from another donor of the same isogenic strain.

These experiments have been successful and tolerance has been induced in this way in all the species tested. The degree of tolerance obtained was extremely variable; in some animals it was complete so that their homografts survived permanently and resembled autografts;

in others the state of tolerance ranged from that which enabled the skin homografts to outlive their controls by many weeks to that which resulted in a prolongation of survival of a few days.

A wide histological variety of tissue cells was found to be capable of inducing tolerance of skin homografts. There was no necessity for the fetal inoculum to include skin epithelial cells, or indeed epithelial cells at all. Tolerance in respect of skin can be induced by the fetal inoculation of whole blood or leucocyte concentrates. Moreover, we have found inocula prepared from embryonic tissues to be effective.

As the cattle work had indicated, tolerance is highly specific; mice which were completely tolerant of skin homografts from one inbred strain reacted with normal vigor against grafts from another, unrelated, strain without prejudicing the well-being of their tolerated grafts.

Tolerance is not due to any kind of antigenic transformation or adaptation on the part of the tolerated graft. A hitherto completely tolerated graft can be caused to undergo prompt regression by the passive transfer of a state of "ready-made" immunity to the tolerant mouse. This is accomplished by implanting it with regional lymph node tissue from another mouse of its own strain which has itself been actively immunized against a skin homograft from the strain which provided the tolerated graft. The tolerant animal is thereby equipped with immunologically active tissue. The susceptibility of the tolerated graft is clearly unchanged; tolerance represents a failure of the host's mechanism of immunological response.

The fertility of tolerant female mice was found to be completely unimpaired, despite the fact that adult male testes were used in the preparation of our standard fetal inoculum.

These experiments may be held to show that tolerance depends upon (a) the fact that embryos are unable to respond to antigens by becoming immunized—a fact which has long been recognized—and (b) a continued inability to respond in later life. Tolerance represents a specific adaptation on the part of the embryo; it is exactly the opposite of "actively acquired immunity" which is the normal response of an *adult* animal to antigens.

The fact that embryos are completely tolerant of foreign tissue grafts has long been known to experimental embryologists; and also the fact that homologous or even heterologous embryonic cells (including melanoblasts, neural crest tissue, limb buds, and skin), implanted very early into avian embryos, may continue to differentiate and survive beyond hatching and even into adult life (see for example Eastlick, 1941;

Rawles, 1945, 1952; Weiss and Andres, 1952). Although the experiments which established these facts were carried out for other purposes, they have strongly hinted at the existence of the phenomenon of acquired tolerance. However, they failed to reveal whether the survival of the foreign cells was the result of an immunological adaptation on the part of the host, or an antigenic change in the grafted cells, or indeed to an immunological process at all.

V. THE INDUCTION OF TOLERANCE IN CHICKENS

Because of the accessibility of their embryos and the fact that these may be injected intravenously, birds are particularly suitable subjects for experiments designed to reproduce more faithfully a situation comparable with that which occurs naturally in cattle twins. The reaction of birds to skin homografts is similar to that of mammals.

The experiments to be described have been carried out with Rhode Island Red (RIR) and White Leghorn (WL) breeds. The main shortcoming of birds for experiments of this kind lies in the absence of strains of full genetic uniformity. The RIR strain available was so heterogeneous that the donor of any fetal inoculation had to be kept for the subsequent test operation.

Cannon and Longmire's work (1952) makes it clear that the epoch of birth represents the upper limit of the tolerance range in chicks, as we have found to be the case in mice; for only 16% of skin homografts exchanged between newly hatched chicks of different breeds gave evidence of prolongation of their survival, and the percentage of successes declined rapidly as the age at which the chicks were grafted was increased. Young birds were therefore not challenged with skin grafts from the donors of their fetal inocula until they were 14 days old. Control experiments established that WL chicks of this age normally reject RIR skin homografts within 9 days.

When 10- or 11-day-old WL embryos were transfused through a chorioallantoic vein with 250 mm^3 of blood from RIR donors of similar age, the majority of those which survived showed some degree of tolerance of their respective donors' skin homografts. The grafts on about 40% of these animals lived beyond 60 days and most of them grew good feather crops of donor specific type. Tolerance has also been induced by transfusing embryos with similar quantities of adult whole blood.

VI. NATURAL SYNCHORIAL TWINNING IN CHICKENS

Nature did not confine her experiments in synchorial twinning to cattle and, rather fitfully, to other mammals (such as sheep (Stormont, Weir & Lane, 1953) and rarely man (Dunsford, Bowley, Hutchison, Thompson, Sanger, and Race, 1951)) for the phenomenon also occurs naturally in chickens. In each of 5 doubly yolked and doubly fertile eggs, the existence of a free anastomosis between the fetal twin circulations has been established. When washed red cells of rabbit were injected into a chorioallantoic vein of one 10- or 11-day-old embryo, they were found in approximately equal numbers in its twin's blood within an hour or two. It was a reasonable presumption from the cattle work that if such twin chicks could be reared they would be red cell chimeras and tolerant of each other's skin homografts. We have succeeded in hatching one pair of such twin chicks. When they were 9 days old their bloods were tested for evidence of red cell chimerism.

This was made possible by the kindness of Dr. R. D. Owen who provided us with a series of lytic antisera prepared in rabbits and suitably absorbed so that they were highly specific for erythrocytes of particular antigenic constitutions. Saline suspensions of the washed red cells under test were treated with these antisera in the presence of guinea-pig complement. The occurrence of partial lysis with any of these antisera was accepted as evidence of the presence of a mixture of two different types of erythrocytes, one of them being susceptible to the lytic reagent concerned.

The tests showed that the twins' blood was an approximately 50:50 mixture. There could be no doubt that these animals were dizygotic.

Skin homografts exchanged between these animals 11 days after hatching are still in excellent condition after 200 days. Besides these animals, another twin bird which was grafted with its partner's skin at birth (for the latter died at hatching) has also proved fully tolerant of its homograft.

These natural twin chicks thus resemble the cattle in being (a) synchorial, (b) chimeras, and (c) tolerant of each other's skin homografts. Thanks to Hašek's (1953; Hašek and Hraba, 1955) technique, which enables a vascular anastomosis to be established between avian embryos experimentally, we have been able to investigate the consequences of synchorial twinning in greater detail.

VII. EXPERIMENTAL SYNCHORIAL TWINNING IN CHICKENS

Discs of shell, about 10-12 mm in diameter, were carefully defined and removed from WL and RIR eggs of 10 days' incubation. After moistening with normal saline, the shell membranes were peeled off to expose the vascular chorioallantoic membranes. The latter were then brought together by the gentle rotation of the eggs, and a lens-shaped plasma clot, containing small fragments of embryonic chick tissue, was inserted between them. This clot was inserted in order to facilitate the establishment of a vascular intercommunication. The junction between the parabiont eggs was then sealed off with paraffin wax.

Despite the high mortality of this method we have succeeded in raising 4 complete parabiotic twin pairs. Interchange of skin homografts between members of the pairs was carried out on the 14th day. All of these animals were highly tolerant of their skin homografts—by the 75th day only one had destroyed its graft—and the donor specific feather crops regenerated by these grafts were, in most cases, quite spectacular. However, as with the cattle twins, evidence of asymmetry of response, i.e. inequalities in the survival times of grafts exchanged between them, was obtained. For example, the graft on animal 287 survived for about 100 days, whereas that on its partner was still perfect on the 245th day. The grafts on 4 of these birds were still in excellent condition when they were finally disposed of after more than 200 days.

Four animals, whose homografts were in perfect condition after about 160 days, were again grafted with skin from their respective partners with complete success.

Fortuitously all 4 complete pairs of parabionts were of dissimilar sex and the normal fertility of all these animals has been established. There is thus no causal connection between tolerance and infertility.

VIII. THE RELATIONSHIP BETWEEN CHIMERISM AND TOLERANCE

All 4 pairs of parabiotic twins were tested for red cell chimerism 40 days after hatching, their skin homografts still being in good condition at the time. It may be added that the life span of red cells in adult chickens is about 28 days (Hevesy and Ottesen, 1945). Clear evidence that their bloods consisted of red cell mixtures was obtained in all cases.

About 4 months later the bloods of two of the pairs, animals 285/286

and 289/290, were again tested for evidence of chimerism. In the first pair, whose grafts had remained in perfect condition, the red cell chimerism remained unchanged. In the second pair chimerism had disappeared completely and their homografts were obviously undergoing a slow deterioration. Breakdown of its homograft was completed 3-4 weeks later in one of these animals, 290, and about 8 weeks later in its partner, 289. These results indicate that chimerism persists only where skin homografts are fully tolerated.

An attempt was made to cross-immunize these two pairs of birds with cross transfusions of blood. Each bird was injected intramuscularly with 10 ml of its partner's blood on 5 weekly occasions. In normal birds of different antigenic constitution this procedure elicits the formation of isoagglutinins (Todd, 1930). In animal 289 no agglutinins developed, despite the fact that its homograft was breaking down and its chimerism had disappeared; in its partner, 290, whose graft had already broken down, a very low isoagglutinin titre was just demonstrable a week after the final injection. Needless to say, the attempt to cross-immunize the twins 285/286, which were fully tolerant and still chimeras, was completely unsuccessful as anticipated, since there was evidence that their bloods were already intermixed.

In summary, this investigation has established that synchorial twinning in chicken embryos results in red cell chimerism and mutual tolerance towards skin homografts in adult life. If the tolerance is incomplete, chimerism and acquired tolerance of homografts will eventually disappear but normal immunological reactivity will not be regained. Finally, red cell chimerism is not accompanied by infertility in twin chicks of dissimilar sex, as it is in cattle. The association of the two phenomena in cattle is presumably attributable to the fact that they have a certain anatomical prerequisite in common—the anastomosis of the fetal blood systems, and possibly the relatively earlier stage in fetal life at which the anastomosis is established.

IX. MATERNALLY INDUCED TOLERANCE

An attempt has been made to investigate whether acquired tolerance may also occur naturally through maternal cells (such as leucocytes) gaining access to the mammalian fetus and so lowering its resistance in later life to skin homografts from the mother. This possibility was strengthened by knowledge of the fate of maternal melanomatous tumors that may rarely gain access to the fetus transplacentally in man (see review by Wells, 1940). Although we have been unsuccessful in

our attempts to demonstrate the occurrence of such a naturally acquired tolerance of maternal skin grafts in mice, cattle, and rabbits, evidence has been obtained suggesting that it may occur occasionally in guinea pigs.

Owen, Wood, Foord, Sturgeon, and Baldwin (1954) looked for evidence of a similarly induced tolerance to *Rh* antigens in *Rh*-negative women born of *Rh*-positive mothers. They found that the chances of *Rh*-negative women being able to produce *Rh* antibody were lessened if they had been born of *Rh*-positive mothers—i.e. if they had had the opportunity of being exposed to the maternal *Rh* antigens in fetal life. However, if instead of antibody formation, the appearance of haemolytic disease in new-born children was used as a criterion of intolerance, no evidence of a relationship between tolerance of the *Rh* antigen and the incidence of the maternal *Rh* type was discovered (see also Booth, Dunsford, Grant, and Murray, 1953).

X. TOLERANCE OF HETEROLOGOUS TISSUES

We have applied the technique of artificial synchorial twinning to study the possibility of inducing "heterologous" tolerance between such remotely related species as chickens and ducks. A series of control experiments showed that skin grafts transplanted from newly hatched chicks to newly hatched ducklings are never accepted by their hosts, even temporarily, as homografts are. Such grafts never became vascularized and were destroyed in less than 6 days. After parabiotically uniting 11-day-old chick embryos with 18-day duck embryos, those which hatched successfully were immediately challenged with their partners' skin. In this way partially tolerant ducks have been obtained which accepted chicken skin heterografts for periods ranging from 8 to 45 days, but none of these grafts ever looked normal; even the most long-lived were chronically inflamed and swollen and never produced any feathers. Evidence of partial tolerance was likewise obtained when skin was transplanted from the ducks to their chick parabionts. This heterologous tolerance seemed to be highly specific, for heterografts from chicks other than their parabionts transplanted to three ducks regressed very promptly.

The low degree of heterologous tolerance obtained in these experiments is probably to be attributed to the fact that the animals were exposed to the heterologous cells too late in embryonic life. Eastlick's (1941) results with limb bud heterografts show that even with 2½-3½ day old donors and recipients complete tolerance is rare. It seems very

probable that an embryo's capacity to respond to different types or classes of cellular and other antigens matures at different rates; in other words, the duration of that phase of embryonic life when acquired tolerance is the outcome of exposure to an antigen depends upon the nature of the antigen concerned. To give only one example, new born cattle react against skin homografts just as vigorously as adults; yet, as Kerr and Robertson (1954) have shown, when very young calves are injected with relatively large doses of *Trichomonas foetus* antigen, their capacity to respond to a later exposure to the same antigen is seriously impaired.

For a full account of the experimental work described in outline in this paper and a comprehensive bibliography on the phenomenon of acquired tolerance see Billingham, Brent, and Medawar (1956) below.

BIBLIOGRAPHY

Anderson, D., R. E. Billingham, G. H. Lampkin, and P. B. Medawar. 1951. The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity 5,* 379-397.

Billingham, R. E., L. Brent, and P. B. Medawar. 1953. 'Actively acquired tolerance' of foreign cells. *Nature 172,* 603-606.

Billingham, R. E., L. Brent, and P. B. Medawar. 1955. Acquired tolerance of tissue homografts. *Ann. N.Y. Acad. Sci. 59,* 409-415.

Billingham, R. E., L. Brent, and P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Phil. Trans. Roy. Soc., B, 239,* 357-414.

Billingham, R. E., G. H. Lampkin, P. B. Medawar, and H. L. Williams. 1952. Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. *Heredity 6,* 201-212.

Booth, P. B., I. Dunsford, J. Grant, and S. Murray. 1953. Haemolytic disease in first-born infants. *Brit. Med. J. ii,* 41.

Burnet, F. M., and F. Fenner. 1949. *The Production of Antibodies.* Macmillan, Melbourne.

Cannon, J. A., and W. P. Longmire. 1952. Studies of successful skin homografts in the chicken. *Ann. Surg. 135,* 60-68.

Dunsford, I., C. C. Bowley, A. M. Hutchison, J. S. Thompson, R. Sanger, and R. R. Race. 1953. A human blood-group chimera. *Brit. Med. J. ii,* 81.

Eastlick, H. L. 1941. Manifestations of incompatibility in limb grafts made between bird embryos of different species. *Physiol. Zool. 14,* 136-143.

Hašek, M. 1953. Parabiose ptáku v embryonálním vývojhi (Parabiosis in birds during embryonic development). *Ceskoslovenká Biol. 2,* 25-27.

Hašek, M., and T. Hraba. 1955. Immunological effects of experimental embryonal parabiosis. *Nature 175,* 764-765.

Hevesy, G., and J. Ottesen. 1945. Life cylce of the red corpuscles of the hen. *Nature 156,* 534.

Kerr, W. R., and M. Robertson. 1954. Passively and actively acquired antibodies for *Trichomonas foetus* in very young calves. *J. Hyg. 52,* 253-263.

Lillie, F. R. 1916. The theory of the freemartin. *Science 43,* 611-613.

Lillie, F. R. 1917. The freemartin: a study of the action of sex hormones in the fetal life of cattle. *J. Exp. Zool. 23,* 371-452.

Owen, R. D. 1945. Immunogenetic consequences of vascular anastomosis between bovine twins. *Science 102,* 400-401.

Owen, R. D., H. R. Wood, A. G. Foord, P. Sturgeon, and L. G. Baldwin. 1954. Evidence for actively acquired tolerance to *Rh* antigens. *Proc. Nat. Acad. Sci. 40,* 420-424.

Rawles, M. E. 1945. Behavior of melanoblasts derived from the coelomic lining in interbreed grafts of wing skin. *Physiol. Zool. 20,* 248-265.

Rawles, M. E. 1952. Transplantation of normal embryonic tissues. *Ann. N.Y. Acad. Sci. 55,* 302-312.

Stone, W., C. Stormont, and M. R. Irwin. 1952. Blood typing as a means of differentiating the potentially fertile from the non-fertile heifer born twin with a bull. *J. Anim. Sci. 11,* 744.

Stormont, C., and R. W. Cumley. 1943. Cellular antigens in cattle blood *J. Hered. 34,* 35-41.

Stormont, C., W. C. Weir, and L. L. Lane. 1953. Erythrocyte mosaicism in a pair of sheep twins. *Science 118,* 695-696.

Swett, W. W., C. A. Mathews, and R. R. Graves. 1940. Early recognition of the freemartin condition in heifers twinborn with bulls. *J. Agr. Res. 61,* 587-624.

Todd, C. 1930. Cellular individuality in the higher organisms, with special reference to the individuality of the red blood corpuscles. *Proc. Roy. Soc., B, 106,* 20-44.

Weiss, P., and G. Andres. 1952. Experiments on the fate of embryonic cells (chick) disseminated by the vascular route. *J. Exp. Zool. 121,* 449-487.

Wells, H. G. 1940. Occurrence and significance of congenital malignant neoplasms. *Arch. Path. 30,* 535-601.

INDEX

aboral halves of *Stentor,* 82-84
Acetabularia, 48, 90
acrasin, 132, 134-136, 142
adaptation, in response physiology, 3-42: level, in light growth response, 11-32; and phototropism, 18; and refractory period, 22; in vision, 35-38; biochemical models, 35, 39-41
adenine, effect on aggregation in *Dictyostelium,* 138
adoral band, *Stentor,* 74, 83
Aerobacter aerogenes, 127
aggregateless mutant in *Dictyostelium,* 129-142
aggregation, cellular, 126; in *Dictyostelium,* 127-151; initiation mechanism, 134, 135, 138-142; and population density, 136, 137; in Fty mutant, 142-147; *see also* synergy
alanine, population control by, 125
algae, symbiotic in *Stentor,* 92, 94-96
Allomyces, 68
all-or-none systems, *see* threshold
Ambystoma, liver RNA, 52, 53; thyroid RNA, 52-54, 56; *A. punctatum,* 157, 200, 208; *A. tigrinum,* 157, 163, 165, 200, 208
angiosperm, *see* apical meristem
antagonism, in *Dictyostelium* mutants, 135, 136
antigens, flagellar in *Salmonella,* 113, 114; red cell, in cattle, 223; in chickens, 227, 228; *Rh,* 230
anura, nervous system, 206, 207
apical meristem of plants, morphogenesis, 174-187: ferns, 174, 175, 178, 179; cycads, 176, 177; angiosperms, 174, 180; isolation, 175; culture, 180, 181; photoperiodism, 181, 182; grafts, 183
Arbacia punctulata oocytes RNA, 47-49
autografts, 221, 222, 224
auxin, in morphogenesis and growth, 176-187; in tissue culture medium, 181
axolotl, 157, 163, 168
axon, *see* nerve fiber

bacteriophage, 106, 108, 111, 112
basal bodies of cilia, 68-70
basophilia, *see* ergastoplasm
Billingham, R. E., 221-232
blood vessels, anastomosis in twinning, 223, 227, 228; hyperneurotization, 203
Brucella abortus, 125
bud primordium of fern, 175, 176; of cycad, 177, 178; of angiosperm, 181, 186

callus, differentiation, 183-186
cambium, 182, 187
cattle, induced tolerance, 222-225, 230, 231
cell, degeneration in neurogenesis, 196, 197, 199, 207, 208; division, unequal, 116, in slime molds, 127, in explants, 160, in plant apex, 174, 178, 181, 186, in nervous system, 192, 193, 199, 207, 208; growth, 45-57; interactions, 126, 136-142, 149-151, *see also* aggregation, induction, synergy; migration, 193, 194; *see also* under specific cell type
central nervous system, 191-209
Chaetomorpha, 68
chemotaxis in slime molds, 136
Chenopodium album, 181
chicken, embryo, neurogenesis, 191-209; extirpation of limb, 196, 197, 208, of eye, 207, of ear, 208; parabiosis, 228; transplantation, 199-202; sciatic nerve, 214; induced tolerance, 226-229, 230, 231; twins, 227-229
chimerae, blood, in cattle, 223, 224; in chickens, 227-229; in *Stentor,* 92-98
Chlorella, see algae, symbiotic
cilia, in plants, 61-70; in protozoa, 62, *see also* cortical pattern; in birds, 62; in mollusca, 64; in amphibia, 64; in mammals, 64; appendages, 64-68; basal bodies, 68-70; dorsiventrality, 64-68; fine structure, 61-70
clam, *see Spisula solidissima*
Coleus, 183
compatibility, nucleus and cytoplasm in *Stentor,* 93-98; self-, in *Escherichia coli,* 102, 103; sexual, in *E. coli,* 103-106
conditioning stimuli in *Phycomyces, see* adaptation level
cortical pattern in *Stentor,* 73-99: morphology, 73-75; polarity, 80-82; symmetry, 80-82; *see also* oral primordium
cytostome, *Stentor, see* oral primordium

deficiencies, morphological in *Dictyostelium,* 128-130
degeneration, cellular, *see* cell degeneration
Delbrück, M., 3-44
deletion, in *Escherichia coli,* 108

development, *see* apical meristem, growth, induction, neurogenesis, slime molds
diatoms, 116
Dictyostelium discoideum, life cycle, 127, 128; mutants, 125-148: Agg, 129, 136, 139-142; Fr, 129-135; Bu, 131, 133, 135; Fty, 142-149
Dictyostelium mucoroides mutants, 128
Dictyostelium purpureum, 138
Dictyota, flagellar spines, 65, 66, 68
Dioon edule, 176
diploidy, in *Escherichia coli,* 108
DNA related to cellular growth and synthesis, 45, 53; in inductor substance, 168, 169; in pneumococcus, 110; in tumor agent, 204
dorsiventrality of cilia, 64-68; of leaf, 175, 176
doublets, *Stentor, see* twins
Draparnaldia, 69
Drosophila, 52
Dryopteris aristata, 175
duck, 230

ectoderm, amphibian, *see* induction
ectoplasm, *Stentor, see* cortical pattern
electron micrography, 46, 53-57, 61-70, 213-219
ergastoplasm, 54-57
Escherichia coli, K-12 strain, 101-121
Euglena, 65, 70
exogenote, 111, 112
explant, amphibian, 157-169; chick, 203-205; plant apex, 174, 176, 181-182

F+ agent in *Escherichia coli,* 103-106
fern, apical meristem, 174-176, 178, 179
fertilization in *Escherichia coli,* 102
fibrils, ciliary, 61-69
fine-line zone, *Stentor,* 74; transplantation, 77-79
flagella, animal and plant, 62-69; *Salmonella,* 113, 114
flowering, induction of, 181
freemartin, 223, 228
frog, midbrain, 207
Fruitless mutant in *Dictyostelium,* 129-135
Fruity mutant in *Dictyostelium,* 142-149
Fruits, in *Dictyostelium,* 128-131, 142, 147, 148
Fucus, cilia, 67-69

Gal locus in *Escherichia coli,* 106, 110-112
galactose, induction of fermentation by, 125
ganglion, chick, bioassay, 203-204; ciliary, 199; spinal, 196, 198, 199, 201, 205; superior cervical, 202; sympathetic, 199, 200-202, 205
genetic particles, formal, 115-117; chromosomal, 117, 118, 120; cytoplasmic, 117-119
Geren, Betty Ben, 213-220
Gingko biloba, 177, 178
Glycine max, 181
grafts, *Stentor,* 76-85, 87-89, 92-99; apex-callus, 183-186; embryonic, 199-202, 208, 224, 225, 230; skin, 222-231
grasshopper, *see Melanoplus, Romalea*
growth, cellular and nucleic acids, 45-57; coupling to illumination and adaptation, 28-32; response to light in *Phycomyces,* 7-42; spiral, 10, 11; vascular plants, 173-187; vertical, 18, 19; zone in Phycomyces, 9, 10
guinea pig, induced tolerance, 230

Hamburger, Viktor, 191-212
Hecht, Selig, theory of light sensitivity, 32-35
heterogenote, 111, 112
heterografts, 230, 231
Helianthus annuus, 180, 181
Hfr genotypes in *Escherichia coli,* 105-107
Himanthalia lorea, 65, 68
histidine, effect on aggregation in *Dictyostelium,* 138
homografts, tolerance, 221-229
hyperplasia, neural, 196, 199-203, 208
hypoplasia, neural, 196-199, 207, 208

immunity, development of, 221-231
induction, embryonic, 149, 155-170, 191
inductor substances, diffusible, 159-170
infection, related to genetic recombinations, 101-115
infraciliature, *Stentor,* 76
inhibition of morphogenesis, *Stentor,* 82-85, 88, 89, 97-99; *see also* antagonism
initiation of aggregation, *see* aggregation, cellular
intensity of stimulus, equivalent, 23, 36; subjective, 28-31
interactions, *see* cell interactions

kappa, in *Paramecium,* 106, 109, 118

lambda, in *Escherichia coli,* 109-111
lamellae, in protoplasmic fine structure, 55-57, 69, 70
lateral geniculate body, 206
leaf of plants, 174-186; dorsiventrality, 175
Lederberg, Esther M., 101-124
Lederberg, Joshua, 101-124

light, growth stimulus and response in *Phycomyces*, 7-42
limb, extirpation, 196-198, 208; supernumerary, 199, 200
Limulus, photoreceptor, 6, 35-38
liver, *Ambystoma*, 52, 53; rat, regenerating, 46-48
locus, genetic, in *Escherichia coli*: Gal, 106, 110-113, Hfr, 106, Lp, 110, 111; in *Salmonella*, H, 114
Lupinus, 176
Lycopodium, 179, 182
lysogenicity, transmission, 108-112

macronucleus, *Stentor*, 74, 75, 90-99
mandibular arch innervation, 200
Manton, I., 61-71
mating types, *Escherichia coli*, 102-108
Mauthner's cell, 206
Melanoplus differentialis, 50, 52
meristem, *see* apical; rib, 180
mesenchyme *in vitro*, 158
micronuclei, *Stentor*, 75, 90
migration, cellular in neurogenesis, 192-194
mitosis, *see* cell division
motor centers of nervous system, 193-200
mouse, sarcoma, 200-202; sciatic nerve, 214; tolerance, 224, 225
muscle spindle, 3, 6
mutants, *see Dictyostelium*; in *Escherichia coli*: Fr, 103-107, Gal, 106, 111, 112, Hfr, 105-107, 110, Lp, 110, 111
Mya, cilia, 64
myelin, compact, 214-217; of central nervous system, 217; sheath, 213-219
myoblasts, 158, 160-162
myonemes, 76

nephros, chick, hyperneurotization, 201-203
nerve, centers: cochlear, 208, lateral geniculate body, 206, mesencephalic V nucleus, 200, 206, neostriatum, 193, 194, nucleus of Terni, 193, optic lobe, 207, 208, spinal cord, 208, 209, trochlear nucleus, 198, *see also* ganglia; fiber, 159, 191-193, 195-209, 213-219; impulse, 3-6
nervous system, *see* induction, neurogenesis
neuroblasts, *see* migration, neurogenesis
neurogenesis, in amphibia, 196, 200, 206-208; in chick, 191-209; effect of periphery, 195-200; humoral factors, 195, 200-206; mechanisms, 191-195; myelination, 213-219; transneuronal factors, 195, 206-209
neuroma in chick, 197-199

Neurospora crassa, 102, 113
Niu, M. C., 155-171
notochord, 158
nucleus, membrane fine structure, 53-57; in *Stentor*, 73-75, 90-99; *see also* basal bodies, DNA, genetic particles, nerve centers, RNA
nucleo-plasmic ratio in *Stentor*, 91
nucleoprotein, in conditioned medium, 164-169; in tumor agent, 204
nucleic acid, *see* DNA, RNA
nucleolus, *see* RNA

Olpidium, 68
oocytes, RNA in, 47-57
optic tectum, transneuronal control, 207, 208
oral primordium, *Stentor*, 74-99
Osmunda cinnamomea, 176, 179
Otala, oocytes, 54-57

parabiosis, *Stentor*, 83-85, 89, 93-97; birds, 228-230
Paramecium, 62, 106, 109, 113, 118, 151
phloem, 178, 184
photoperiodism, 178, 181
photosynthesis and growth, 173
phototropism in *Phycomyces*, 11, 14-20
Phycomyces blakesleeanus, light growth response, 7-42
phyllotaxy, 174
pigment, cells, 158-160; photosensitive, 32-35; stripes in *Stentor*, 73-74; *see also* fine-line zone
pneumococcus, 110
polarity, *see* apical meristem, cortical pattern
pollen, *Tradescantia*, 48, 49, 52
populations, microbial, proportions, *see* synergy; variation, 126
position effect, 112, 113
posterior medullary plate (PMP) as inductor, 158-169
prophage, 109-111
protein, synthesis, 45, 57; in inductor, 164-169; in tumor agent, 204
pseudoallelism, 112, 113

rabbit, induced tolerance, 224
range adjustment, *see* sensitivity
Rasch, Ellen, 45-59
rat, RNA in liver, 46-48; induced tolerance, 224
Rebhun, Lionel, 45-59
recombination, genetic in *Escherichia coli*, 101-112
red cell chimerism, 223, 224, 227-229

regeneration, *Stentor*, 76-99; rat liver, 46-48
Reichardt, W., 3-44
replication, genetic particles, 119-121
Rh antigens, tolerance, 230
RNA, chromosomal, 49-52, 53; in inductor substance, 168, 169; nuclear-cytoplasmic relations, 45-57; nucleolar, 46-49, 51, 52; perinuclear, 53
Romalea microptera, 51-53

Saccheromyces chevalieri, 125, 142
Salmonella, 106, 110-114, 116
Saprolegnia, 65
sarcoma, mouse, 200-202
Schwann cell, relation to axon, 213-219
Scitosiphon, 68, 69
sea urchin, *see Arbacia*
segregation, genetic, *Escherichia coli*, 106-108; *Salmonella*, 116
Selaginella, 174, 179
sensitivity, range in *Phycomyces*, 20-32; related to photoreception, 32-38; physical interpretation, 38-41; to lysogenicity, *Escherichia coli* K-12, 109
sexual reproduction in microorganisms, 102; *see also* mating types
shoot, in plants, development, *see* apical meristem; floral, 178; lateral, 178; long vs. short, 176, 177, 186
skin grafts, 222-231
slime molds, 125-151
specificity of nucleus and cytoplasm, *Stentor*, 92-98; of induced tolerance, 225
spermatogenesis, RNA in, 51, 53
Sphagnum, cilia, 61, 64
spinal cord, neurogenesis, 192, 193, 196-200
spinal ganglia, *see* ganglia
Spisula solidissima oocytes, 48, 49, 54-57
sporangiophore, *see Phycomyces*
Stentor, morphology, 73-76; transplantation, 76-99; minced, 86-87; fused masses, 87-89; *see also* chimerae, parabiosis; *S. coeruleus*, 73-98; *S. felici* N. Sp., 76; *S. niger*, 92, 97, 98; S. *polymorphus*, 92, 94-97; Sp. *X*, 92, 93
streptomycin, effect in *Escherichia coli*, 107
subjective intensity in light growth response, 28-31; stimulus, 29-31
Sussman, Maurice, 125-154
Sussman, Raquel R., 125-154
Swift, Hewson, 45-59
symmetry, cilia, 65-68; cortical pattern in *Stentor*, 80-85
synapse in development, 195, 202
synergy of development in *Dictyostelium* mutants, 130-136, 144-147; failure, 132, 135, 136; mechanisms, 133-136, 147
Synura, flagella, 64, 65, 67-69
Syringa, grafts, 183-185

Tartar, Vance, 73-100
teleost optic tectum, 207
threshold response systems, 4-6
thyroid, amphibian, 52-54, 56, 206
tissue culture, conditioned medium, 155-160, 163-169; plants, 174, 181, 182; bioassay, 203-205
tolerance, tissue, 221-231; and fertility, 223, 228; to heterografts, 230, 231; to homografts, 221-230; maternal induction, 229-230
Tradescantia paludosa pollen, 48, 49, 52
transduction, 101, 102, 106, 108, 110-112, 114
transneuronal effect in neurogenesis, 195, 206, 208, 209
transplantation, *Stentor*, 76-99; angiosperms, 183-185; embryonic, 196, 199-202
triplets, *Stentor*, 78, 84
Triturus torosus, 157, 162, 163, 166, 168, 169; *T. rivularis*, 157, 162, 168, 169
tumor agent, in neurogenesis, 195, 200-203, 205; chemical characterization, 203-205
twins, bird, 227-231; cattle, 222-224; *Stentor*, 77-83, 88, 93-98

urodele nervous system, 196, 199

vascular, connections in twins, 223, 224, 226-228, 230, 231; plants, growth, 173-187; tissue, differentiation, 182; *see also* apical meristem, phloem, xylem
Vaucheria, 68, 69

Wetmore, R. H., 173-190
Woodard, John, 45-59

Xanthium saccharatum, 181
Xiphophora chondrophylla, 65
xylem, 178, 183, 184

yeast, 113; *see also Saccharomyces*