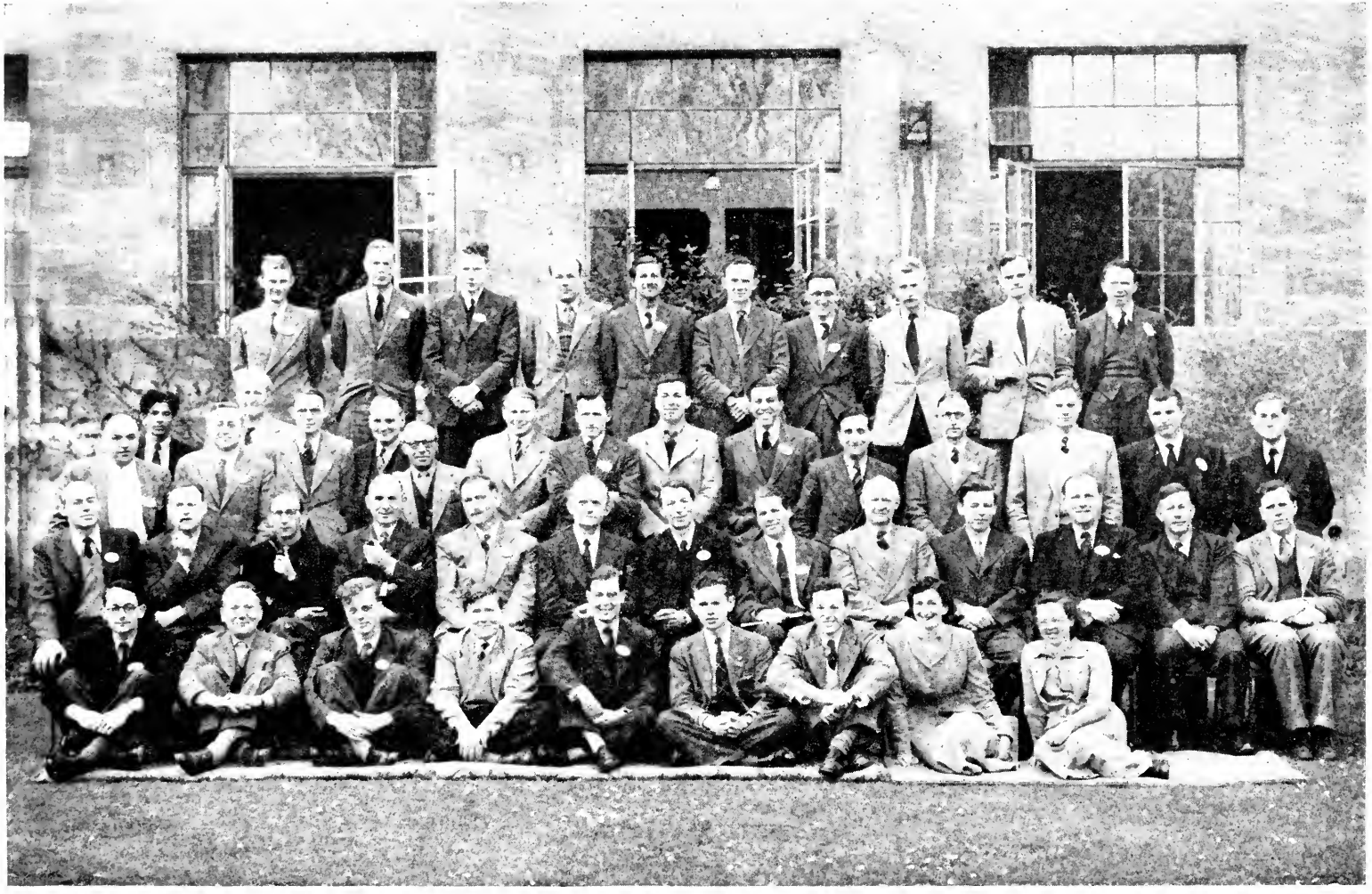
RECENT DEVELOPMENTS IN CELL PHYSIOLOGY



The Members of the Symposium

RECENT DEVELOPMENTS IN CELL PHYSIOLOGY

Edited by

J. A. KITCHING

Proceedings of the
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Vol. VI. 1953 *Insecticides and Colonial Agricultural Development*—Editors, Professor T. Wallace and Dr. J. T. Martin.

There will be no Colston Symposium in 1955, owing to the visit to Bristol of the British Association. The series will be resumed in 1956.

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Foreword

It was in 1899 that a group of public-spirited Bristol citizens established the 'University College Colston Society', whose chief aim was to assist the then young and struggling University College. The Society was named after the noted seventeenth-century philanthropist and educationalist, Edward Colston. The Annual Dinner of the Society soon came to be regarded as a function of considerable importance in the life both of the University and of the community as a whole.

It was at the Society's dinner in 1908 that the public announcement was made of the gift of £100,000 by H. O. Wills to the University. The period of expansion which was ushered in by this gift resulted finally in the granting of a Charter, and the attainment by the University College of full university status. At this time too the Society changed its name, became the 'Colston Research Society', and decided to direct all its energies to the promotion of research. For twenty years it collected annually an average sum of over £600 which was devoted to this end. In the decade from 1929 to 1939 the activities and resources of the Society underwent considerable expansion, and it not only continued to make research grants to University departments, but it also financed at considerable cost a social survey of Bristol.

However, with the further rapid growth of the University in the post-war period it became clear that the financial contribution of the Society was becoming less and less important in relation to the very large funds which were needed by University departments for their research work. Accordingly the Society decided once again on a radical change of policy and resolved to devote the major part of its funds to the promotion of an annual symposium.

The rapid growth in popularity of the symposium as a means for the advancement and stimulation of knowledge is one of the remarkable features of the intellectual life of recent years. For this development there have been a number of interesting and compelling reasons, all of which the Society carefully considered before embarking on its new policy. This policy has already achieved a remarkable measure of success; it has been a pioneer effort among the universities of Great Britain, and represents a distinctive contribution on the part of Bristol University to the cultural life of the country as a whole.

A list of the subjects of the six previous symposia appears on the opposite page. It will be seen that in arranging these symposia it is intended that they shall be free to cover all fields of learning, provided that they are not too highly specialized, but possess a reasonably wide appeal, and are at a sufficiently interesting stage of development to make it likely that they will benefit by symposium treatment.

As President of the Society for the year 1953–54 it was my privilege to preside over the seventh symposium, on 'Recent Developments in Cell Physiology'.

H. C. I. Rogers.

Preface

CELL physiology to-day is the common meeting-ground of the botanist and zoologist, of the biochemist and biophysicist, of the geneticist and embryologist. In spite of the ambitious field which we have attempted to cover in fifteen short papers, no excuse is needed for bringing together students from so wide a range of disciplines to present some of their latest work, to discuss their separate and common interests and to speculate on the future of this fascinating subject. How far this aim has been successful is only partly to be judged on the contents of this present volume; the many informal groups collected between the official meetings are not the least valuable feature of any symposium of this kind.

For financial and other reasons, the geographical range of the speakers was more restricted than their academic one. The Society's guests from overseas included only workers from Denmark and Belgium, both countries which have made great contributions to cell physiology in recent years.

In order to assist the discussion, papers were roughly grouped so that each session dealt with a similar general topic.

The opening session was concerned with the exchange of material between the cell and its environment, with particular reference to the mechanisms of active transport. This topic was continued on the second day by papers on membrane structure and on the ionic permeability of the nerve fibre. The metabolism of the cell and certain special problems of nucleic acid synthesis were represented by three papers, and the study of the nucleus was then broadened to include its role in the metabolism of the cell and morphogenesis of the organism. A particularly interesting session on the external synchronization of cell division was followed by a final meeting in which the control of differentiation and of cell division were considered as well as some new physical properties of the cell surface in Protozoa.

The contributors to the symposium are particularly to be congratulated on the broad treatment of their subject-matter, which stimulated discussion and speculation in the friendly and informal atmosphere which was so characteristic of the whole meeting. To this atmosphere our Danish and Belgian guests brought a spontaneity and good fellowship which was only equalled by their amazing facility in the English language.

My own position as Director of the symposium has been an unexacting one of privilege without responsibility. Dr. J. A. Kitching has taken the whole burden of editing the manuscripts and the discussion, and thanks to him and to the co-operation of all the participants, this volume has been produced in a surprisingly short time. Our indebtedness to the printers and publishers is equally obvious, and gives me the opportunity of paying tribute to the help of Mr. R. H. Brown, who, in his other capacity as Secretary of the Colston Research Society, has been as invaluable as on

earlier occasions. The administrative arrangements for the meeting were in the capable hands of Dr. H. P. Whiting, who, with Miss Morgan, Warden of Manor Hall, and her staff, was in no small part responsible for the success of the symposium.

Finally, the separation of the foreword from this preface gives me a very welcome opportunity, on behalf of all my colleagues, to pay our tribute to the President, Mr. H. C. I. Rogers and to the Colston Research Society for sponsoring this symposium, and for all they have done, and continue to do, for the University of Bristol.

J. E. Harris.

Bristol, 1954.

Contents

The present position in the field of facilitated diffusion and selective active transport

by

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DEFINITION AND CHARACTERIZATION OF PROCESSES

It is desirable to distinguish, as accurately as is possible at the present time, between several processes: diffusion, facilitated diffusion, and selective active transport. This contribution is concerned with *selective* active transport, i.e. active transport which is selective for a limited range of molecular species. It is not concerned with unselective active transport; for instance a process whereby environmental fluid is accumulated unchanged in a vacuole on one side of a membrane, and discharged unchanged from the vacuole on the other side of the membrane, would be active transport, but unselective and therefore not of significance in this discussion.

Diffusion is brought about by the driving force of thermal agitation. In a homogeneous fluid the rate at which a given molecular species diffuses may be calculated, at constant temperature and pressure, if the viscosity of the fluid and the molecular weight of the diffusing species are known*. As a result of a diffusion, the free energy of the system is lowered, and there is usually a decline in gradients of chemical potential† if no force other than that of thermal agitation is acting upon the molecules (i.e. if gravitational, electrical and other forces have no significant effect upon the final distribution of molecules). Thus diffusion is selective in terms of molecular weight (or linear dimensions†), but is unselective in terms of structural and steric factors.

Facilitated diffusion also occurs under the driving force of thermal agitation, but differs from diffusion in that the rate at which molecules diffuse is strongly influenced by structural and steric factors. It is a process commonly found in studies of the permeability of plasma membranes, and in the past has usually been included in the category of active transport. But it is better separated as a special type of diffusion, since the equilibria attained by facilitated diffusion are the same as those achieved by diffusion. The difference between the two processes is essentially that, by facilitated diffusion, some molecular species may reach diffusion equilibrium much more rapidly than would be possible by non-facilitated diffusion.

* This is true for molecules of a molecular weight of up to about 1,000. When the molecules diffusing are very large compared with the solvent molecules, the rate of diffusion is more accurately calculable from the linear dimensions than from the molecular weight.

† This is not always so, for if the diffusion of two species is linked, as in Osterhout's well-known guaiacol model for K^+ accumulation, there may be an increase in the chemical potential gradient of one species achieved at the expense of a decline in the gradient of another species.

Active transport involves the movement of molecules by forces additional to those of thermal agitation. The result of active transport may (if the free-energy change of the active process is ignored) be an increase in free energy of the system, and an increase in chemical potential gradients.

Of these three processes we know least about active transport, and most about diffusion. As we learn more about the nature of active transport, it may prove that a number of distinct types of process are involved which can be defined separately, just as it has recently proved possible to separate facilitated diffusion from active transport.

CLASSIFICATION OF TECHNIQUES

A variety of techniques may be employed to differentiate between diffusion, facilitated diffusion and active transport, and to approach the mechanisms underlying individual processes of facilitated diffusion and active transport. These techniques can be roughly divided into six groups.

(1) *Morphological studies.* The examination of the structure and ultrastructure of secretory organs and cells is of outstanding importance, and too frequently underrated. In many cases the limits of analysis using the light-microscope are far from exhausted. The exploitation of electron-microscope studies should be of the greatest value, as is suggested by the recent publication of Sjöstrand's (1953) work on the free and other cell borders, nuclear membranes and mitochondrial membranes of kidney proximal tubule cells. It is probable that a full understanding of the details revealed by electron-microscopy must await the development of cytochemical methods for use with electron-microscopy, but even to have available the structural detail, without chemical detail, of the membranes concerned will be highly stimulating.

(2) *Kinetic studies.* The examination of the rates of penetration of substances through membranes, and of the effect of variation in concentration, temperature, ionic strength and other environmental conditions on these rates, are included in this heading. Included also is the effect of variation in molecular structure and stereochemistry, and the use of isotopes. Isotopes are particularly useful in the determination of total transfer, as opposed to net transfer.

(3) *Metabolic studies.* Although studies of metabolism are unlikely to yield much useful information about the actual mechanism of transfer, they are often useful in showing that transfer is *in some way* dependent on metabolism. This permits distinction between diffusion on the one hand, and facilitated diffusion and active transfer on the other. It does not necessarily permit distinction between facilitated diffusion and active transfer, since in the former case, although no energy contribution is required from the cell for transfer, energy may be required to maintain the membrane in an active state permitting facilitated diffusion. The effect of metabolism is usually best studied by depriving the cells of metabolites (e.g. glucose) or by use of poisons (e.g. cyanide, dinitrophenol).

(4) *Cytochemical studies.* These are at present sharply limited by the lack of a sufficient variety of reliable cytochemical techniques. A few observations of major importance have been made, including: (*a*) the high concentration of alkaline phosphatase at the secretory surfaces of many secretory cells (Danielli, 1953); (*b*) the high

concentration of cholinesterase associated with the membrane of motor end plates (Holt, 1954); the high concentration of periodate-oxidizable carbohydrate at the surface of secretory cells (Ruyter, 1953; Bell, unpublished).

(5) *Direct activators and inhibitors.* It is thought probable, on somewhat slender grounds, that a number of substances act directly on transfer mechanisms, e.g. phosphate esters and acetylcholine (Danielli, 1953), insulin and anterior pituitary hormone (Cori, 1945), phloridzin (Rosenberg and Wilbrandt, 1952), dinitrofluorobenzene (Bowyer, 1954) and possibly some of the oestrogens (Bullough, 1953). It is to be hoped that more substantial evidence bearing on these interactions will soon become available.

(6) *Potential studies.* Where movement of ions is involved, the selective movement of any one species will result in formation of an electrical potential difference. Such potential differences must be compatible with, and quantitatively explained by, the movements of the individual ions. Consequently, potential measurements have a valuable place in transfer studies although, as Gasser (1933) stated, 'you cannot determine a process from a potential'.

EXAMPLES ILLUSTRATING THE PRESENT PROBLEM

There have been several recent reviews, e.g. Rosenberg and Wilbrandt (1952), Goldacre (1952), Danielli (1953), Stadie (1954) and the recent S.E.B. Symposium (Volume VIII, 1954). In this symposium Dr. Koch and Dr. Keynes will be concerned with movements of ions: I shall limit myself to non-electrolytes.

(*A*) *The penetration of sugars into muscle*

When a substance is injected into an animal it rapidly becomes distributed through the blood and extracellular spaces, but the extent to which it penetrates into intracellular water is determined by ability to pass through cell plasma membranes. Table I summarizes some of the main results obtained by Levine and his colleagues (1950, 1953*a*, *b*). These results were obtained on animals which had been eviscerated and nephrectomized, so that side effects due to metabolism and excretion might be minimized. The data contained in the table effectively outline the problem as it presents itself in mammals. Urea, which readily enters most mammalian cells, is distributed in a volume of water equivalent to 70 per cent. of the body weight (i.e. practically all the body water), whereas the non-penetrating substance sucrose is distributed in an equivalent of 45 per cent. weight. Insulin has no effect on the distribution of either sucrose or urea. *d*-glucose is initially distributed in 45 per cent., and there is an increase on adding insulin. But results with glucose are complicated by metabolism, and the insulin effect is better seen with *d*-galactose, *l*-arabinose and *d*-xylose, with which a distribution-weight of 45 per cent. is raised to 70 per cent. by insulin. *l*-rhamnose and *d*-arabinose are not metabolized, and show no insulin effect, and *d*-fructose, *d*-mannose and *l*-sorbose, though metabolized, show very much less effect of insulin than does glucose. Thus insulin enables some substances to penetrate readily into a volume of body water into which they move with great difficulty, in the absence of insulin. The insulin action is structurally and sterically specific—e.g. is positive for *l*-arabinose and negligible for *d*-arabinose. Also it is not

TABLE I

The distribution of injected substances in the water of eviscerated nephrectomized animals. The figures are for the equilibrium volume of water in which the substances appear to be distributed, expressed as percentages of body weight. It is assumed that at equilibrium the concentrations of substances in all water into which they penetrate are the same as the concentrations in the blood.

Substance	Whether metabolized	Percentage of body weight occupied	
		no insulin	with insulin
urea	−	70	70
sucrose	−	45	45
d-glucose	+	45*	⩾45
d-galactose	−	45	70
l-arabinose	−	45	70
d-xylose	−	45	70
l-rhamnose	−	45	45
d-arabinose	−	45	45
d-fructose	+	all metabolized, but show relatively little effect of insulin	
d-mannose	+		
l-sorbose	+		

* Followed by slow steady increase, due to metabolism.

linked with metabolism, for it is positive with some non-metabolized sugars and negligible for some sugars which are metabolized.

Results of similar significance to those of Levine *et al.* have also been obtained with rat diaphragm (e.g. Haft, Mirsky and Perisutti, 1953) and heart (e.g. Bleehen and Fisher, 1954). It has been shown that the insulin effect on glucose penetration may be antagonized by other hormones, including anterior pituitary 'growth' hormone and cortisone, and it is thought to be simulated by oestrogens (see, e.g. *Ciba Foundation Colloquia*, VI). The relationships between the various hormones are far from understood, for Ottaway (1951) has shown that 'growth' hormone, normally antagonistic to insulin, under some circumstances has an insulin-like action. It is possible that the anti-insulin action of 'growth' hormone involves displacement of insulin from the plasma membrane by the hormone, and that the insulin-like action is due to 'growth' hormone releasing active insulin by displacement from an inert tissue complex (Ottaway, 1953).

Another observation of great interest is that (Bleehen and Fisher, 1954) when a rat heart, with coronary circulation, is perfused with an insulin-free medium, insulin is removed from the heart at about the same speed as is the polysaccharide inulin.

The removal of both compounds has a 'half-life' of about four minutes. The speed with which insulin can be removed suggests that its action must be upon the external surface of the plasma membrane. The alternative hypothesis, that there is a very rapid interchange of insulin across the plasma membrane, whilst not utterly impossible, is highly improbable.

It has been suggested (Cori, 1945) that these hormones act upon hexokinase, and that penetration is a phosphorylative transfer catalysed by hexokinase. The above results are not readily explicable on this hypothesis.

The penetration of glucose into muscle cells is commonly described as active transfer. However, there is no evidence that movement of glucose into muscle cells ever occurs against a concentration gradient. The simplest hypothesis compatible with the observations recorded to date is that glucose enters muscle cells by facilitated diffusion, that it is phosphorylated after entry so that a concentration gradient favouring entry is maintained, and that the action of insulin is exerted from the cell exterior upon the mechanism of facilitated diffusion.

(*B*) *Penetration into human red cells*

Most non-electrolytes enter red cells by simple diffusion, but there are a number of exceptions to this rule. Urea enters many red cells abnormally fast—probably by facilitated diffusion: the same is true of glycerol for the red cells of many rodents and primates, and of glucose for the red cells of primates. Glycerol penetrates about 10^2 times faster into human red cells than is calculated should be the case for simple diffusion, and as is found experimentally for many cells, including cattle, sheep and pig red cells. Glucose penetrates about 10^4 times faster than is calculated. But neither of these substances is caused to move against a concentration gradient. These substances penetrate at a 'normal' speed through most of the cell surface, and abnormally fast through a small fraction of the surface (Danielli, 1943). These small active patches are often readily poisoned—e.g. by copper ions (Jacobs and Corson, 1934). When the active patches are poisoned, the rate of penetration falls to that calculated for diffusion. Davson (1954) has recently suggested that the high rate of penetration found with anions is also a case of facilitated diffusion.

On the other hand, several investigators, particularly Maizels (1954), have shown that K^+ and Na^+ may move into and out of red cells by active processes, against concentration gradients. Deprivation of glucose stops the active process and movement of ions then occurs, by diffusion, down the concentration gradients.

Table II sets out some of the results which have been obtained with enzyme poisons on the movements of glucose, glycerol, sodium and potassium with human red cells. Copper ions and bromacetophenone strongly inhibit facilitated diffusion of both glycerol and glucose, whereas iodoacetate does not. These results suggest that there may be SH groups concerned, but relatively unreactive SH groups since iodoacetate is ineffective. But dinitrofluorobenzene, although a very powerful inhibitor of facilitated diffusion of glucose, does not inhibit glycerol movement. This is compatible with involvement of SH groups in the case of glucose, but not in the case of glycerol. Diazonium hydroxides have no effect with either glycerol or glucose, so that it is possible that neither tyrosine, histidine or tryptophane are involved. The inactivity of iodoacetate, cyanide and dinitrophenol shows that respiration, glycolysis

and phosphorylative oxidation are not immediately involved. Phlorizin, phloretin and phloretin phosphate are active, and are alkaline phosphatase inhibitors, suggesting that alkaline phosphatase is concerned. Phloretin phosphate is colloidal, and inhibits mainly *exit* of glucose from the red cell.

In the case of sodium and potassium movements it is known that glycolysis, not respiration, supplies the energy. But in the case of the (nucleated) red cells of the hen, Maizels (1954) has shown that respiration provides the energy for active transport. If the respiration of hen red cells is poisoned, glycolysis continues but is unable to

TABLE II

The action of enzyme poisons upon penetration into human red cells

Poison	Facilitated Diffusion		Active transfer
	Glucose	Glycerol	Na and K
Pb, Cu, etc.	+	+	(+)
Bromacetophenone	+	+	
Iodoacetate	−	−	
Phlorizin	+	+	
Phloretin	+	+	
Phloretin PO_4	+	+	
Cyanide	−	−	−
Dinitrophenol	−	−	
Dinitrofluorobenzene	++	−	delayed effect
Diazonium hydroxide	−	−	++
Eserine	−	−	delayed effect

actuate active transport. This suggests that some form of coupling is necessary between the energy-yielding process and the mechanism of transport. The fact that glucose as a source of energy may be replaced by acetylcholine, and that the transport mechanism may be poisoned with eserine, no matter what source of energy is used, suggests that cholinesterase is concerned in the mechanism of transport (Hollander and Grieg, 1950). The failure of phlorizin, phloretin and phloretin phosphate to inhibit active transport of K^+ and Na^+ appears to exclude alkaline phosphatase.

The general conclusion which emerges from these studies is that enzyme-like membrane components appear to be active both in facilitated diffusion and active transport. The detailed examination of the composition of red cell membranes should clearly be pursued. Reviews bearing on this problem have been published by Brown (1952) on plant cells and by Rosenberg and Wilbrandt (1952) on animal cells.

A PRELIMINARY ANALYSIS OF THE MEMBRANE PROCESS

(1) *Membrane morphology*

A variety of evidence indicates that the plasma membrane is basically a lipoid layer about 50 Å thick, with protein layers adsorbed on either side, i.e. a sandwich structure (Harvey and Danielli, 1934, 1938; Danielli and Davson, 1934; Danielli, 1942). In a number of instances this conclusion has been checked by electron-microscopy (Sjöstrand and Rhodin, 1953): from the electron-microscopy studies it appears that the total membrane thickness is about 200 Å of which about 50 Å are lipoid sandwiched between two protein layers each about 70 Å thick. In 1933, when this sandwich structure was first proposed, it was suggested that the protein components consisted of at least one monolayer of adsorbed unfolded proteins, with a secondary adsorbed layer of globulin. There appeared to be no way in which protein could be incorporated in the lipoid layer, to make a mixed membrane of mosaic structure (Danielli, 1936). Recent developments in the examination of protein structure make it possible to modify this view.

In the case of haemoglobin, and the same is probably true of many other globular proteins, the structure consists of lamellae. Each lamella has one hydrophobic and one hydrophilic surface, and the lamellae are paired so that in aqueous solution the hydrophobic surfaces are back to back and the protein-aqueous interface is thus mainly hydrophilic. These pairs of lamellae may further associate, specifically, in sets of two or more pairs (Fig. 1*A*). Such associations are fairly stable, but may be broken by hydrogen-bonding substances such as urea.

In general, if an aqueous pore were opened in a lipoid membrane, surface-tension forces would tend to enlarge the pore, and in the absence of a restraining force would destroy the membrane. If, however, the pore is a slit between two protein lamellae, as indicated in Fig. 1*B*, the same attractions which, in aqueous solution, serve to hold together the hydrophilic surfaces of two haemoglobin lamellae, in the membrane may withstand the low surface-tension forces tending to enlarge the pore.

Thus from consideration of what is known of plasma membrane morphology, and from the known properties of lipoid molecules, we can envisage a structure which, whilst lipoid to a first approximation, as is known to be the case for many cell membranes, has a limited number of polar pores.

(2) *The rate of permeation at constant temperature*

Table III shows experimentally determined permeability constants for various non-electrolytes entering into human erythrocytes and the cells of *Chara ceratophylla*, compared with the calculated permeability constants for a membrane composed of 50 Å of hydrocarbon with a viscosity of 10^5 times that of water. Within permissible error, the values for *Chara* are in agreement with the calculated values, and so are the values for human red cells, with three exceptions—those for urea, glycerol and glucose—which are very much larger than the calculated values. Thus from consideration of rates of permeation it may be shown that the membranes of many cells are, *to a first approximation*, homogeneous lipoid layers. But there are a few substances which do not fit into this hypothesis. It can be shown, both by calculation and by

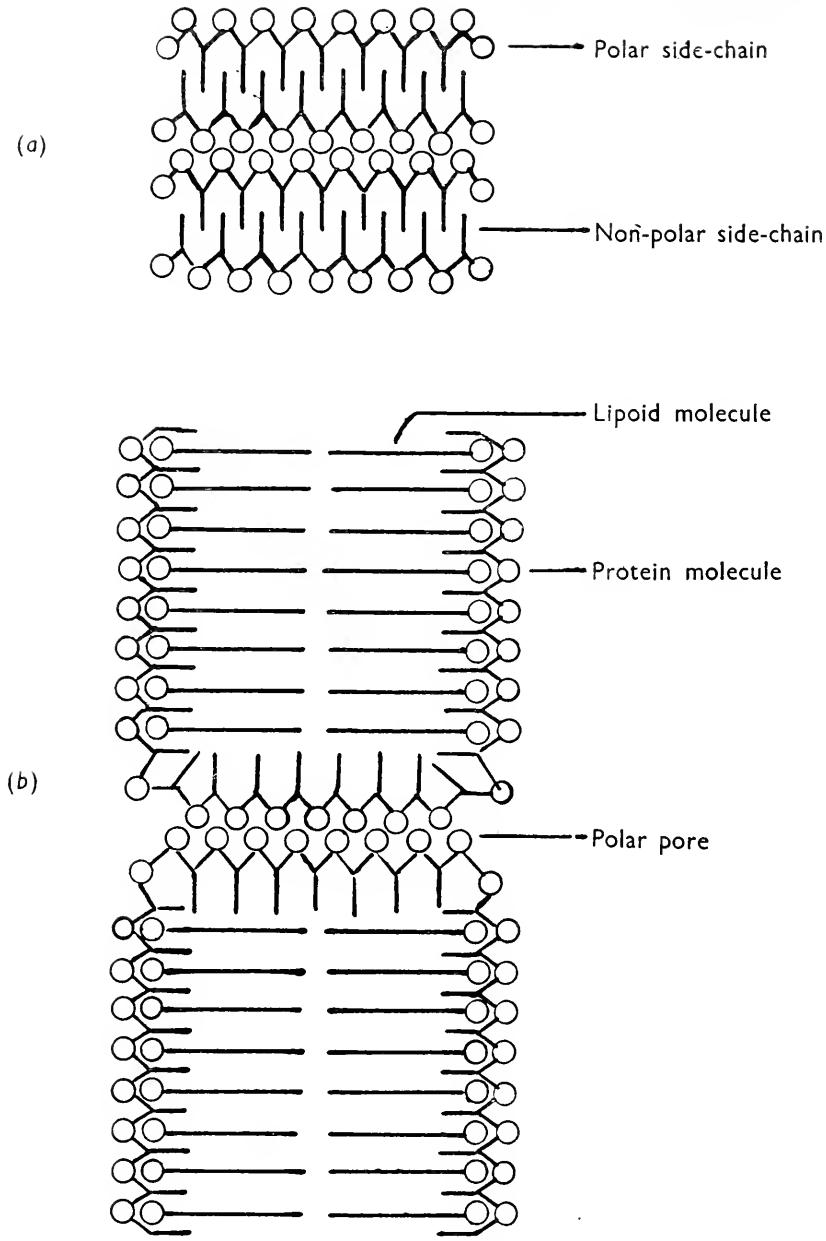

Figure 1. (*a*) *Diagram of protein molecule;* (*b*) *Diagram of pore of membrane.*

experiment, that only a small fraction of the total surface area is involved in permitting the abnormally high rate of penetration of these exceptional substances.

Recently the work of Collander (1949), of Jacobs (1952) and of Ussing and Zeuthen (this symposium) has shown that very small molecules, such as methyl alcohol,

formamide, water, and urea, frequently penetrate cells faster than is compatible with permeation through a strictly homogeneous lipoid layer. In this group of molecules the only specific feature seems to be that the molecules must be very small, and hydrogen-bonding. With the occasional rapidly permeating large polar molecules, such as glucose, mentioned in the previous paragraph, the specific features are ability

TABLE III

Comparison of calculated and experimental permeability constants. All values multiplied by 10^{16}

Substance	Calculated for 50 Å hydrocarbon	Observed values	
		Chara	Human red cell
Propionamide	3·0	3·6	~3·0
Glycol	0·8	1·2	0·2
Urea	0·02	0·11	**8·0**
Glycerol	0·005	0·02	**0·1**
Erythritol	0·0001	0·001	0·0003
Glucose	0·000001	—	**0·1**
Sorbitol	0·00000004	—	<0·0001

to form hydrogen bonds and a very sharp structural specificity: for example, the exceptional rate of entry of glucose into human red cells is not shown by either methyl glucoside or sorbitol.

(3) *The effect of temperature on permeability constants*

When the change of permeation rate with temperature is considered, it is again found that the membranes of many cells are homogeneous lipoid membranes, *to a first approximation*. And again, the same molecules which were exceptionally fast in penetrating, by comparison with other molecules, provide evidence of inhomogeneity in the membrane.

Thus glycerol penetrating ox red cells has a permeability constant of 0·002 and a Q_{10} of 3·5, both of the order of magnitude to be expected for glycerol permeating a homogeneous lipoid layer. But glycerol permeates rabbit red cells much more rapidly, with a permeation constant of 0·05 and a Q_{10} of 2. When the special mechanism which permits this is poisoned with a trace of Cu^{++}, the permeability constant falls to about 0·002 and the Q_{10} rises to 3·5. The values for the poisoned membrane show that its general structure is that of a homogeneous lipoid layer, but that this is normally masked, in the case of glycerol, by a small proportion of areas highly and specifically permeable to this substance.

It is also found that the very small molecules which penetrate abnormally fast also have abnormal Q_{10} values.

Thus the accumulated evidence from kinetic studies shows that:

(*a*) plasma membranes are homogeneous lipoid membranes, to a first approximation.

(*b*) very small molecules penetrate more rapidly than would be expected for a lipoid membrane, and their permeability constants have anomalous temperature coefficients.

(*c*) some larger polar molecules penetrate more rapidly than expected, and their permeability constants have anomalous temperature coefficients.

(*d*) for larger molecules abnormally rapid penetration is shown by a very limited range of molecular structures.

(*e*) only a small proportion of the total plasma membrane area displays permeability properties which would not be expected of a homogeneous lipoid layer.

(4) *The nature of the membrane process*

To approach the problem of the mechanism of abnormally rapid penetration, we must first state the mechanism of normal permeation of a lipoid layer. This may be considered to involve three steps.

(*a*) *Entry into the membrane*: this involves breaking the hydrogen bonds linking a molecule to water, the overcoming of van der Waals' forces, and the formation of a hole in the membrane large enough to accommodate the penetrating molecule.

(*b*) *Diffusion through the membrane*: this involves mainly the overcoming of van der Waals' forces between hydrocarbon chains and between hydrocarbon chains and the diffusing molecules.

(*c*) *Exit from the membrane*: this involves processes the reverse of (*a*).

Any one of these three steps will prove limiting for an appropriate molecular species; e.g. for highly polar molecules such as erythritol, (*a*) is limiting because of the number of hydrogen bonds which must be broken before the molecule can break away into the membrane. For acetamide, steps (*a*) and (*c*) are relatively unimportant, so that the viscous resistance encountered in diffusing through the membrane becomes the limiting factor. With octyl alcohol the number of hydrogen bonds involved is relatively small, but on leaving the membrane a large hole must be formed in the water to accommodate the eight CH_2 groups: this requires a good deal of energy and hence step (*c*) is limiting.

In view of these facts, how can we account for abnormally rapid permeation?

For very small molecules an explanation could be, and perhaps often is, found in the existence of more than one phase in the lipoid layer (Danielli, 1949). We can envisage that very small molecules may be able to penetrate through all the various lipoid phases, whereas larger molecules would be able to pass through only the less densely packed molecules. This, however, does not explain how a very polar molecule such as glucose may penetrate abnormally fast.

Abnormally rapid penetration of a polar molecule such as glucose can occur only if a special mechanism is provided for breaking its hydrogen bonds with water, e.g. if the membrane contains a carrier molecule which will form hydrogen bonds with the polar molecule, so that the complex between the two forms no hydrogen bonds with water. This would not offer any very satisfactory explanation of why very small

molecules may penetrate abnormally fast. An alternative explanation is that a hydrogen-bonding proton-conducting component extends right through the thickness of the membrane. One can readily conceive that such a structure would be selectively permeable to a limited range of hydrogen-bond-forming molecules such as glucose, and also permeable to many small hydrogen-bond-forming molecules.

(5) *The contribution made by enzyme studies*

Various lines of evidence, involving the use both of enzyme poisons and of cytochemical methods, have indicated that alkaline phosphatase and cholinesterase, or substances of similar properties, are often concerned in abnormally rapid penetration processes. There are theoretical reasons for treating this information with reserve, for if the structures permitting facilitated diffusion and active transport are hydrogen-bonding proton-conducting components, with structural and stereochemical specificity, we might well expect them to exhibit specific enzymic activity which is incidental* and not concerned in the permeation process (Danielli, 1954*a* and *b*). But for the purposes of this symposium I shall assume that these enzymes are in fact directly concerned.

AN ATTEMPT AT A SYNTHESIS

If the data and considerations presented above are brought together to present a general picture of the plasma membrane we must take into consideration:

(*a*) the 'sandwich' structure of the membrane;
(*b*) its approximation to a homogeneous lipoid layer;
(*c*) that abnormal permeabilities may be explained if in some areas a polar structure extends right through the membrane;
(*d*) that enzymes are present at the sites of transfer, as shown by cytochemical methods;
(*e*) that poisons for these same enzymes selectively block transfer;
(*f*) some enzymes are known to provide the mechanism whereby chemical energy may be used to activate a contractile protein mechanism;
(*g*) to facilitate permeation of polar molecules, hydrogen bonds between the molecules and water must be broken: this can be done by supplying protons or alternative hydrogen-bond-forming groups;
(*h*) hydrolytic enzymes, such as phosphatases and esterases, probably work by providing a stereochemically specific hydrogen-bonding proton-conducting surface (just as the non-specific hydrolytic catalysis characteristic of ionic resins and ionic colloidal micelles is probably due to their non-specific proton-conducting surfaces);
(*i*) so far as can be seen, the specificity for certain molecules, both of enzymes and of transfer processes, must depend upon the same organization of groups in space, both with respect to their nature and their critical spacing and orientation.

All the above points are provided for if we adopt the hypothesis that facilitated diffusion involves movement through a pore or slit composed of the polar groups of protein lamellae, as in Fig. 1*B*. The junction between two protein lamellae will not be a simple aqueous pore: it will be a region composed of polar groups and including

* Just as the esterase activity of certain peptidases is probably incidental.

a good deal of water, as is the case with protein crystals, and extensive hydrogen bonding between the protein chains will give it a unique character. The properties of this polar pore will include:

(i) ready permeability to small hydrogen-bond-forming molecules such as water and formamide.

(ii) if the protein component is positively charged, e.g. if it were haemoglobin, it would be selectively permeable to small anions, and thus provide the facilitated diffusion mechanism in red cells suggested by Davson. If negatively charged it would be selectively permeable to small cations.

(iii) to larger polar molecules the pore would be permeable only if the structure and configuration of the molecule conformed to the structure of the pore.

(iv) passage through such a pore need not occur by movement of the penetrating molecule only. We can envisage the protein components of such pores oscillating between different configurations. Examples of such oscillations are found in reversibly denatured proteins. Such oscillations may assist in conveying molecules through the membrane.

(v) a pore of this nature offers a basis for working out possible modes of action of hormones, such as insulin and 'growth' hormone, which are concerned in transfer processes.

(vi) a pore of this character provides a mechanism which will permit proteins to pass through plasma membranes. The possibility of such passage would depend upon the specific configurations of the proteins of the pore and of the permeating protein, and a mechanism of this type may account for selective permeability to proteins of the type reported by Brambell and Hemmings for the passage of antibodies through the intestinal wall, etc.

(vii) pores of this character would not only exert the selectivity characteristic of facilitated diffusion, but would also be susceptible to the action of enzyme poisons, such as those mentioned in Table II.

In short, a pore structure of this type appears to provide an excellent working hypothesis for study in connexion with facilitated diffusion. The components of the pore need not be entirely restricted to protein, but might include nucleic acids, polysaccharides, etc. This conception has the additional advantage that by simple extension the mechanism of facilitated diffusion becomes a mechanism of active transport. Where movement of the penetrating species is determined by the kinetic energy of the penetrating molecules themselves, or by the oscillation of a protein between alternative structures under the influence of thermal agitation, the process is facilitated diffusion. But if the movement is determined by a contraction-expansion, or oscillation, impressed upon a protein by the energy released by the enzymic action of that protein, then we have active transfer. Thus Goldacre's (1952) concept of the importance of contractile proteins in active transport becomes logically connected with the mechanism deduced for facilitated diffusion.

As was mentioned earlier, the permeation of glucose which is insulin-dependent need not be active transfer but may be facilitated diffusion, with glucohexokinase present at the inner end of the facilitating pore. Alternatively, hexokinase might be one of the pore proteins, with the phosphorylative mechanism at the inner end of

the pore and the insulin-sensitive mechanism at the outer end. If this were so, an explanation would be provided for the insulin effect upon hexokinase action being dependent on the preservation of structure.

Many other problems—particularly the phenomena of conduction of impulses by excitable cells—need to be considered from the point of view advanced here, but this must await another occasion.

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Cholinesterase and active transport of sodium chloride through the isolated gills of the crab Eriocheir sinensis *(M.Edw.)*

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INTRODUCTION

A NUMBER of freshwater insects and Crustacea share with Amphibia and fishes the ability to transport mineral ions from very dilute solutions into a much more concentrated blood (see Krogh, 1939).

From the point of view of general cellular physiology it certainly is a fortunate circumstance that the structures responsible for this uptake in arthropods are composed of only one single layer of cells. In the most favourable cases specialized cells are assembled so as to form definite organs. The function of this type of organ has been established on firm ground at least in the case of the anal papillae of Diptera (Koch and Krogh, 1936; Koch, 1938; Wigglesworth, 1938; Krogh, 1939; Ramsay, 1953).

Recently it has been possible to show that the gills of the freshwater crab *Eriocheir sinensis* (M.Edw.) will continue to absorb ions when isolated from the body. This active transport occurs against a considerable concentration gradient (e.g., outside medium 8 mM Na, blood up to 300 mM; Koch, Evans and Schicks, 1954*a*).

By means of these gills it is now possible to study an ion-absorbing mechanism in an arthropod without the interference of other parts of the body. These gills have already proved to be promising material for the analysis of ion uptake, and their considerable size makes them suitable for the further investigation of the biochemical basis of active transport. Their homogeneous histological composition gives them in this respect an advantage over the isolated frog's skin, which has contributed so much to fundamental knowledge of active transport (Huf, 1935; Krogh, 1937; Ussing, 1948; Ussing and Zerahn, 1951).

The mechanism by which NaCl is actively taken up by the gills of the crab shows some remarkable features in common not only with the corresponding function in the larvae of Diptera but even with ion-transport mechanisms in vertebrates.

GENERAL CHARACTERISTICS OF THE ION ABSORPTION

The ion-absorption mechanism present in the gill epithelium is able to work at high speed: values of 0·2 mg. NaCl per gill per hour have been observed repeatedly with

a well-aerated 8 mM external solution. If we take into account that such a gill contains about 2·5 mg. of nitrogen, corresponding at most to 15·6 mg. of protein, we obtain 2·5 mg. NaCl/g. tissue/hour. This figure is quite impressive for the performance of such a gill, especially when we remember that the centre of the gill is not active under such circumstances.

Fast and steady absorption is recorded only on freshly prepared gills; the speed of absorption declines slowly (Figure 1). After three or four hours a condition is usually reached in which no net intake is observed. However this only means, in the terminology used by Ussing (1948), that outflux counterbalances influx, as may be shown by

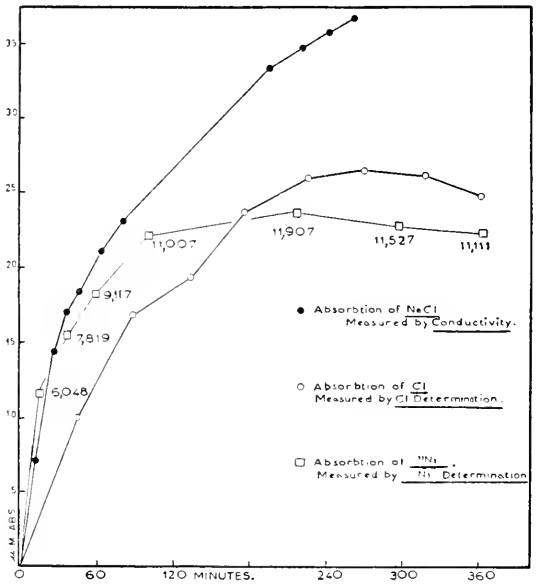

Figure 1. *Time-course of the absorption of Na, Cl, and NaCl from an* 8 mM *NaCl solution as determined on three different gills of a crab.* (*From Koch, Evans and Schicks*, 1954*a*.)

means of ^{22}Na used as tracer. The outflux continues independently of the simultaneous influx, as will be seen from the following experiment, which also shows the influence of CO_2 (Figure 2). A gill is allowed to absorb NaCl from a relatively strong radioactive solution containing ^{22}Na, the total concentration of NaCl being 8 mM. After thirty minutes or so it is transferred immediately, after careful washing, to a non-radioactive solution of the same strength (concentration of NaCl, 8 mM; volume of solution used, 12 cc.). In the latter solution the absorption measured by conductivity continues. At a certain time the air is replaced by a mixture of air and CO_2 so that the influx of NaCl stops. The outflux continues and can be measured by means of the outwardly diffusing tracer. When pure air replaces CO_2, a strong influx again takes place. Taking into account the total NaCl content of the gill at the end of the experiment, it becomes possible to calculate the absolute magnitude of the outflux.

During the period of treatment with the CO_2 and air mixture, there is a close correspondence between the estimates of outflux obtained by the tracer and by the conductivity methods, so that it seems rather probable that the increase in conductivity is fully accounted for by the outflow of NaCl. It is also clear that the net intake represents the difference between a considerable outflux and a normally still more considerable influx of Na.

The inhibiting effect of CO_2 on the ion-absorption mechanism has been described by Ussing for the frog's skin, and we have found that the same substance is also a very strong inhibitor for the sodium uptake of the anal papillae of the larvae of Diptera.

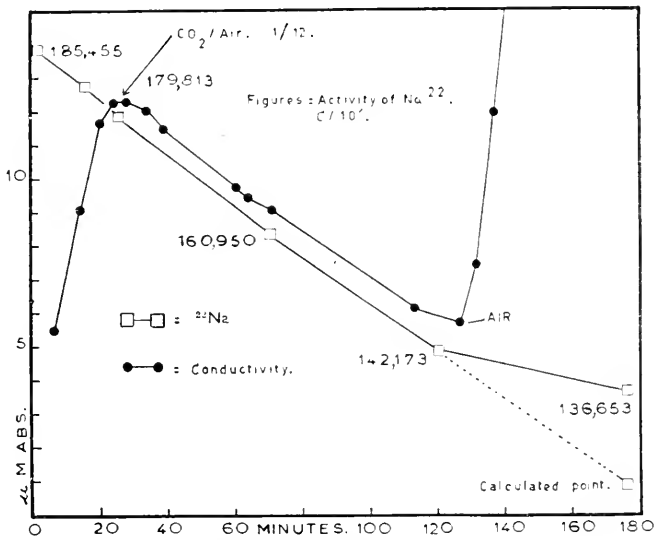

Figure 2. The reversible inhibition by CO_2 (1 part in 12 of air) of the net uptake of NaCl (as determined by conductivity). Simultaneously the outflux of previously absorbed ^{22}Na is measured. (From Koch, Evans and Schicks, 1954a.)

In the complete absence of oxygen, only outflux takes place. The necessity of free oxygen for ion uptake in *Chironomus* larvae has been shown already by Hers (1942).

Classical inhibitors of cytochrome oxidase (KCN, NaN_3), previously used by Krogh (1939) on whole *Eriocheir*, as well as Na_2S, have a strong depressing effect on ion absorption: the reversibility of their influence is easy to show on isolated gill preparations.

It is tempting to imagine that the need for cytochrome oxidase, so well established for wheat roots by Lundegardh (1951), is general for all ion-absorbing systems. However this enzyme seems to play no part in the ion-absorbing mechanism of the anal papillae of *Chironomus* larvae (H. Koch and Schicks, unpublished).

While trying to elucidate the importance of the cytochrome-cytochrome oxidase system for ion absorption in the gills of the crab, it seemed interesting to investigate what would happen in the presence of a substance able to open a new pathway for

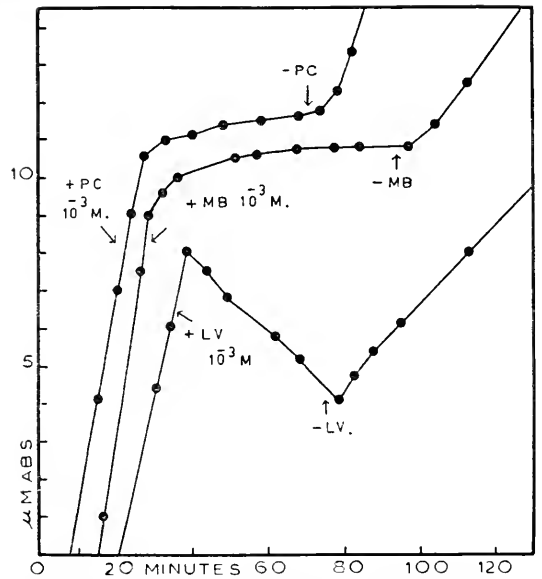

Figure 3. Influence of the action of different basic dyes (at 10^{-3} M*) on the absorption of NaCl from an 8 mM NaCl solution (as determined by conductivity). The dyes are added at + and washed away at —. (After Koch, Evans and Schicks,* 1953*, corrected scale.)*

P C: pyocyanine; M B: methylene blue; L V: thionine.

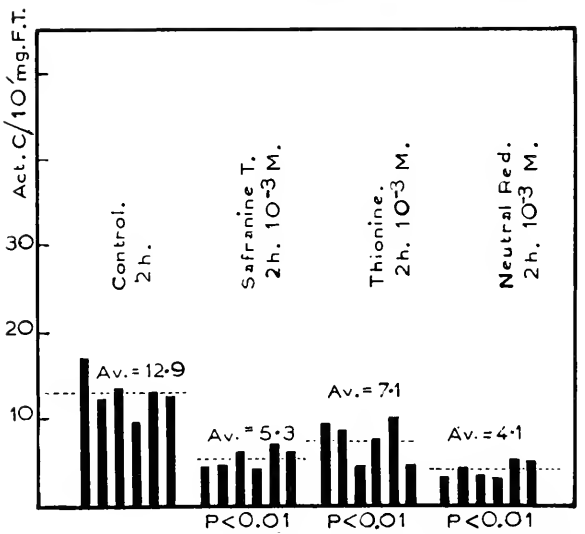

Figure 4. Influence of different basic dyes on the Na absorption by the anal papillae of the larvae of Chironomus plumosus. *The relative amounts of Na absorbed after the same time are expressed on the basis of activity per milligram fresh tissue per* 10 *minutes. (Koch, Evans and Schicks,* 1954*b.)*

the activated hydrogen. Accordingly the effects of well-known hydrogen carriers like methylene blue and pyocyanine were tested, and they proved to be excellent inhibitors of salt absorption (Figure 3) not only in the gills of the crab but also in the anal papillae of *Chironomus* (Figure 4). Their action is rapid and consistent and, after washing the gills of the crab, their effects proved to be completely reversible.

TABLE I

Chemical group	Name of Dye	E'_o	Concentration	Result
		BASIC DYES		
Azines	Neutral red	−0·340	10^{-3} M	Slow-working inhibitor
	Safranine T.	−0·289	10^{-3} M	Reversible inhibitor
	Pheno-safranine	−0·250	10^{-3} M	Reversible inhibitor
Indamines	Bindschedler's green	+0·224	10^{-3} M	Reversible inhibitor
	Toluylene blue	+0·115	10^{-3} M	Reversible inhibitor
Oxazines	Brilliant cresyl blue	+0·047	10^{-3} M	Reversible inhibitor
	Ethyl Nile blue	−0·122	10^{-3} M	Reversible inhibitor
Phenazine	Pyocyanine	+0·034	10^{-3} M	Reversible inhibitor
Thiazines	Methylene blue	+0·011	10^{-3} M	Reversible inhibitor
	Thionine Lauth's violet	+0·062	10^{-3} M	Reversible inhibitor
		ACIDIC DYES		
Indophenols	2, 6, Di-Cl-phenol-indo-phenol	+0·217	10^{-3} M	Non-inhibitor
Oxazines	Gallocyanine	+0 021	10^{-3} M	Non-inhibitor
Sulphonphthaleins	Phenol red	−0·340	10^{-3} M	Non-inhibitor
Thiazines	Alizarin blue	−0·173	10^{-3} M	Non-inhibitor

The facts seemed at first to be consistent with the idea that these substances act as inhibitors because of their oxidation-reduction properties. Working further on this assumption we tested different dyes in order to determine the limits of their activity in relation to their position on the rH scale. Discrepancies appeared rapidly, substances as far apart as Bindschedler's green ($E'_o = +0{\cdot}224$) and safranine T ($E'_o = -0{\cdot}289$) both being active.

A list of the dyes so far tested is given in Table I; they belong to very different chemical groups. When we looked for characteristics common to these widely different substances, it appeared that all the inhibitors of salt transport were basic dyes, whereas the inactive ones were acidic dyes (Koch, Evans and Schicks, 1953.)

However the mere fact that a substance of nearly the same molecular weight as these dyes was a basic compound was not enough to confer on it an inhibiting action: substances like quinine proved to be inactive.

The only further salient feature of the dye inhibitors was the presence of a quaternary NH_4 group. When tetramethyl ammonium chloride was tested it also proved to be an inhibitor of salt transport.

Now some quaternary ammonium compounds at least are known as inhibitors of cholinesterases, and the cholinenergic properties of methylene blue on the heart of vertebrates has been described by Heymans (1923) and R. P. Cook (1926). From the purely biochemical point of view the anti-cholinesterase activity of basic dyes has been investigated by Rentz (1940) and especially by Massart and Dufait (1941) on horse-serum cholinesterase. The latter authors have shown that the anti-cholinesterase activity of basic dyes is dependent on the quaternary NH_4, because it disappears in the leuco-form where N is no longer present as a quaternary compound.

All this suggested that the inhibitory action of basic dyes on salt transport might be due to their influence as anti-cholinesterases. However such an interpretation of the inhibition of salt transport by means of basic dyes required further evidence, especially because basic dyes have been also described as inhibitors of dehydrogenases (Quastel and Wheatley, 1931).

In order to elucidate the effect of basic dyes on the respiratory activity of the gills, experiments were conducted with Warburg manometers in which the dyes could be tipped in from a side arm at a certain moment. The oxygen consumption was measured with pure O_2 and with an 8 mM solution bathing the gills. The gills were prepared exactly as for an active transport experiment: they were ligatured at the base.

While acidic dyes had no effect on respiration, basic dyes increased the respiratory intensity (Safranine T from 30 to 126 cu. mm. per hour). Therefore the inhibition of active transport was not caused by a depression of respiration.

THE PRESENCE OF CHOLINESTERASE AND ITS LOCATION IN THE GILL EPITHELIUM

A suspension of the gills of *Eriocheir sinensis* (obtained by means of three minutes' treatment in 25 cc. of bicarbonate Ringer in a mixing blender) clearly exhibits cholinesterase activity when tested in the presence of acetylcholine according to the method of Ammon.

Measurements of the activity showed that 1 g. of fresh gill tissue is able to hydrolyse

43 mg. of acetylcholine per hour. This figure compares favourably with the figure of 5 to 50 mg. given by Nachmansohn (1952) for nerve fibres.

Eriocheir gill cholinesterase is inhibited by means of basic dyes. Acidic dyes have comparatively speaking little effect, as is apparent from Figure 5.

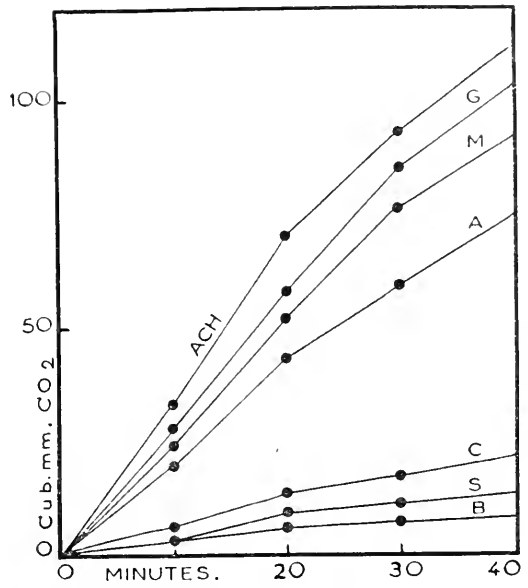

Figure 5. Evolution of CO_2 as a consequence of the splitting of acetylcholine under the influence of a fine suspension of gill tissue in the presence of basic and acidic dyes. (Koch, Evans and Schicks, 1954b.)

ACH: control with acetylcholine alone,
G: in the presence of gallocyanine (acidic dye),
M: ,, ,, ,, ,, methyl blue (acidic dye),
A: ,, ,, ,, ,, alizarine blue (acidic dye),
C: ,, ,, ,, ,, crystal violet (basic dye),
S: ,, ,, ,, ,, safranine T (basic due),
B: ,, ,, ,, ,, basic fuchsin (basic dye).

The blood of the crab itself contains a cholinesterase which is inhibited by means of the same basic dyes as well as other cholinesterase inhibitors. When the blood is expressed as far as possible from the gills before the mixing process a considerable cholinesterase activity remains. It thus seemed highly probable—but not certain—that cholinesterase is present in the gill epithelium itself.

Basic dyes were then injected into the crab in such concentration as to reach approximately 10^{-3} in the blood; and the gills, deeply stained from the inside, were afterwards tested for active transport. The gills so treated proved to be as active absorbers as normal gills. In this way it became evident that the cholinesterase of the blood, completely inhibited by the presence of the dye, was of no importance for the active absorption process. It also seems hard to escape the conclusion that cholinesterase is present along the exterior surface of the gill epithelium and that it is this

cholinesterase which is concerned in the active transport of ions. Even if cholinesterase is also present on the inner surface of the gill epithelium this will not help to explain the effect of basic dyes on the outer surface of the gills; this inwardly-situated cholinesterase would also be out of action in the last-mentioned experiment because of the anti-cholinesterase effect of the injected basic dyes.

INFLUENCE OF CLASSICAL INHIBITORS OF CHOLINESTERASE ON THE ABSORPTION MECHANISM OF THE GILLS

The foregoing interpretation of the action of basic dyes is corroborated by the effect of classical inhibitors of cholinesterases such as eserine (physostigmine) and diisopropylfluorophosphate. Their inhibitory influence on salt transport is shown in Figure 6. The inhibitory action of these substances is entirely reversible.

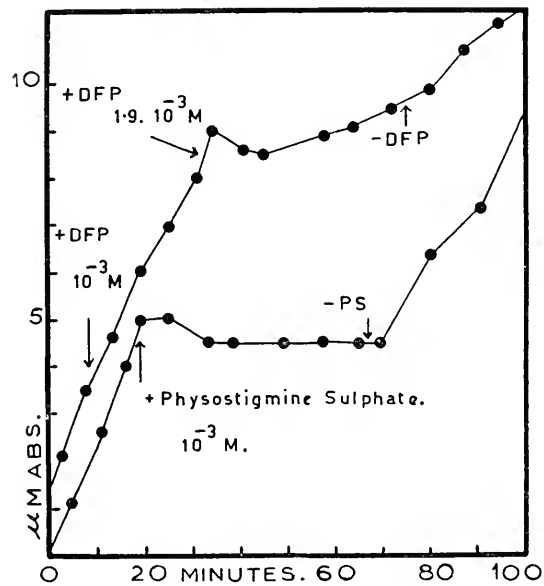

Figure 6. NaCl absorption by a gill of the crab as influenced by DFP (diisopropylfluorophosphate) and eserine (physostygmine). Upper curve: DFP added first at 10^{-3} M *concentration, and later, in the absence of effect, brought to* $1 \cdot 9 \times 10^{-3}$ M. *DFP removed at –DFP. Lower curve: eserine added at* 10^{-3} M *concentration, and removed at –PS. (From Koch, Evans and Schicks, 1954b.)*

Tetraethylpyrophosphate is generally considered to be an irreversible inhibitor of the cholinesterases, and we also found (Koch, 1954) that its inhibition of salt transport is irreversible after washing with water. Kirschner (1953) made a similar observation on frog skin. However Wilson (1951, 1952) has shown that the inhibition of cholinesterase with alkyl-phosphates is reversible under the

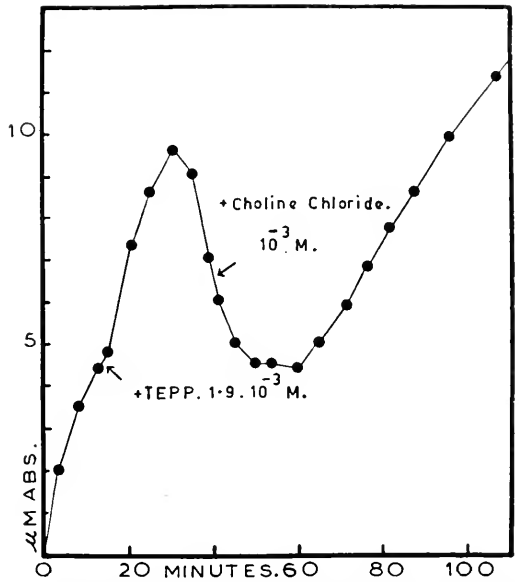

Figure 7. Influence of TEPP (tetraethylpyrophosphate $1{\cdot}9 \times 10^{-3}$ M*) on the NaCl absorption of a gill of the crab and its reversal on the further addition of choline chloride* 10^{-3} M.

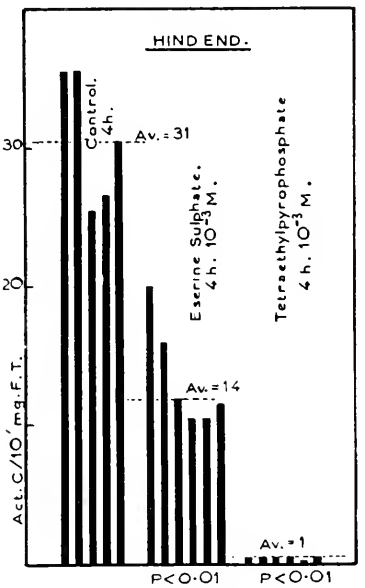

Figure 8. Absorption of ^{22}Na *(expressed on basis of activity per mg. of fresh tissue) by the isolated hind ends (3 segments) of the larvae of* Chironomus plumosus *influenced by eserine* (10^{-3} M) *and tetraethylpyrophosphate* (10^{-3} M) *as compared with a control. (Koch, Evans and Schicks,* 1954*b.)*

influence of choline and other substances. Recently we were able to obtain also with choline a reversal of the inhibition of salt transport by TEPP for the gills of *Eriocheir* (Figure 7). All these inhibitors have an entirely similar effect on the absorption of Na by the anal papillae of the larvae of *Chironomus* as illustrated by Figure 8.

INFLUENCE OF CURARE

It may be of special interest to notice that *d*-tubocurarine hydrochloride, which is supposed to act selectively on neuromuscular transmission, is also quite an active inhibitor of ion transport in the gills of *Eriocheir*. Tubocurarine is supposed to act

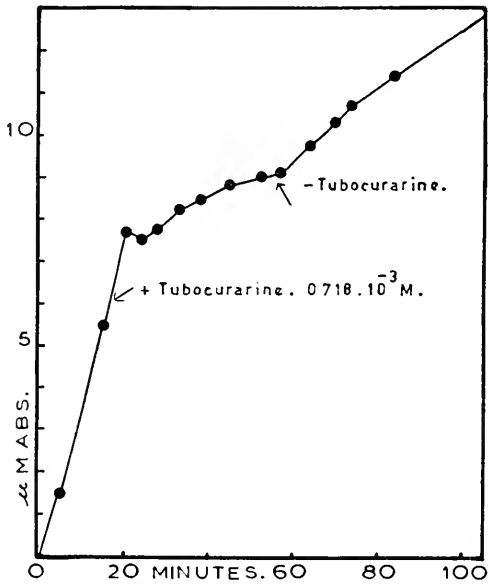

Figure 9. Influence of d-*tubocurarine HCl on the NaCl absorption of a gill of a crab. (Koch, Evans and Schicks, 1954b.)*

not so much by inhibiting cholinesterase as by competing with acetyl choline for the same receptor protein. This action seems to be partly reversible (Figure 9). In certain cases it has been shown that cholinesterase inhibitors affect the permeability of these structures (Rothenberg, 1950).

CONCLUSIONS

The most suggestive fact which seems to emerge from what has been explained so far is the importance of a cholinesterase as a component of the mechanism which actively transports ions through the gill epithelium of the crab. Moreover a cholinesterase seems to be also an important part of the mechanism for the active transport of Na in the anal papillae of an insect, the larva of *Chironomus plumosus*.

Now it has been shown recently by Kirschner (1953) that an enzyme which belongs to the same group is a part of the mechanism for the active transport of Na in

the frog's skin. Besides all this the acetylcholinesterase has long been known to play a part in the transmission of nerve impulses at the motor end plates of muscles and in synaptic transmission, and it probably also takes part in conduction along the nerves of many species of animals (Fessard and Posternak, 1950; Nachmansohn, 1950). We thus find this same enzyme associated with phenomena which in certain of their aspects are intimately associated with active transport of the ions of alkali metals (Hodgkin, 1951; Rothenberg, 1950).

The action of acetylcholine and cholinesterases on the heart muscles of vertebrates and invertebrates is well known (Krijgsman, 1952), and quite recently Bülbring,

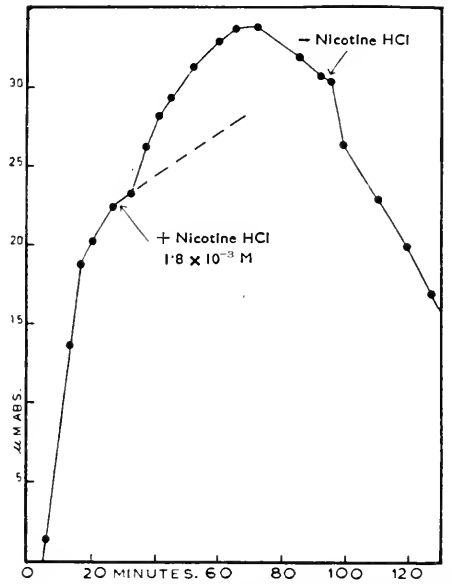

Figure 10. *Nicotine hydrochloride first accelerates but soon depresses the absorption of NaCl by the gill of* Eriocheir. *Removal of the inhibitor does not restore the active uptake.*

Burn and Shelley (1953) have shown that cholinesterase and acetylcholine are also essential for the ciliary movements of *Mytilus*.

All this seems to suggest that these widely different mechanisms are dependent on some common basic cell activity which may turn out to be an active transport phenomenon, applied in hypertrophic condition, if one may so describe it, for certain functions.

We do not intend to dwell on further details or to collect a multiplicity of data which do not lead to any further elucidation. Nevertheless I think it is worth while to mention the effect of nicotine (Figure 10) on ion transport in the gills of the crab. It is an inhibitor, but before it inhibits it clearly accelerates the rate of absorption of NaCl. Is this not what from another point of view vertebrate pharmacologists would call a nicotinic effect? Undoubtedly there are big differences as far as effective

dosage is concerned, but let us not forget that the external organs of arthropods are protected by a cuticle which certainly restricts the access of larger molecules to the underlying sensitive cell surface.

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Discussion

ON PAPERS BY (1) J. F. DANIELLI AND (2) H. J. KOCH

Chairman: J. E. Harris

O. Maaløe. Would it be possible to estimate the number of the hypothetical 'aqueous pores' so accurately that it could be said whether such structures could be expected to show up in electron-micrographs such as have been produced by Sjöstrand and his colleagues?

J. F. Danielli. There are various methods which give a numerical value for an apparent 'aqueous' pore, such as the kinetics of penetration or the amount of Cu^{++} required to poison facilitated diffusion. These values agree in indicating that the total area of the pores is small—probably less than 1 per cent. of the surface; but I hesitate to place any reliance on the absolute magnitude of the figures.

R. J. Goldacre. What are the relative merits of the hypothesis of protein molecules contracting through pores in the membrane and that of the complete folding up of the membrane and its solution in the cytoplasm together with any molecules adsorbed onto it from outside? It seems that relatively few fibres could pass through a pore, so that the same contraction pulling these fibres through would tend to cause folding up of the membrane as a whole owing to the contraction of adjacent fibres not passing through pores. Also would you not expect that the existence of pores sufficiently large to allow significant amounts of electrolytes to get through would require an electrical conductivity for the plasma membrane considerably different from that found?

J. F. Danielli. Facilitated diffusion may occur without active folding of a protein or its oscillation between two states. It is sufficient for the protein to provide a polar channel through the membrane. Facilitated diffusion would be converted into active transport by the development of contractility of the protein. Folding of the whole membrane, and its dissolution, may occur in some instances, but in others, for instance the erythrocyte, such a process is most unlikely. The electrical conductivity of the plasma membrane is higher than would be expected for a homogeneous lipoid layer, so that the existence of a limited member of ion-permeable pores is not incompatible with the membrane conductivity.

R. D. Keynes. Have you any views as to how protein pores might achieve the remarkable discrimination exhibited by cell membranes between anions and cations or between sodium and potassium?

J. F. Danielli. I should, in general, expect discrimination between sodium and potassium to be possible by a mechanism involving the formation of co-ordination compounds. The stereochemical properties of a co-ordinating molecule could be such as to give a considerably greater stability to the compound with sodium than the compound with potassium, or vice versa.

R. J. Goldacre. Instances are known where the absorption of potassium is considerably different from that of sodium. For example, Szent-Gyorgi and his co-workers have isolated various myosin-like proteins from kidney, heart, brain, lung, and muscle, which are characterized by a relatively high absorption power for potassium ions; for myosin itself I think he found that 1 g. of potassium was absorbed by about 4,000 g. of myosin. Also in the related field of absorption on mineral surfaces, potassium and sodium may behave quite differently. This difference is exploited in the commercial separation of NaCl from KCl by the froth flotation process.

W. S. Reith. Concerning the preferential position of the potassium ion I should like to mention that a nitrated polystyrene ion-exchange resin has been described in the literature which possesses an unusual affinity for the potassium ion.

N. Myant. How does the aqueous pore theory fit in with penetration of protein molecules to the inside of cells?

J. F. Danielli. Proteins may in theory penetrate through polar pores. Whether they will do so or not will depend upon the nature of the pores and of the proteins. One would expect the process to be highly selective.

N. Myant. Discrimination between sodium and potassium need not depend on specificity of pores. It might be due to an intracellular reversible complex formed with potassium but not sodium.

J. F. Danielli. So far no intracellular complex has been found which is highly selective for sodium or potassium.

M. M. Swann. I should like to draw attention to the parallel which seems to exist between active transport and the fertilization of an egg by a sperm. Fertilization is a highly specific process of course, and there is little doubt that the sperm is drawn passively through the egg membrane. I have also been struck by the fact that many of the substances which have a powerful effect on active transport also affect fertilization. Copper and lead, and most basic dyes, inhibit it; nicotine induces polyspermy. Work that Rothschild and I have done suggests that one can actually put a figure on the probability of a successful sperm-egg collision, and we are inclined to think of the reaction in immunological terms. Would it help to think of active transport in these terms?

J. F. Danielli. It is possible that the reason why there is a similarity between factors influencing active transport and factors influencing fertilization is that in both cases the phenomena are mediated by contractile proteins, as Goldacre (1952, *Int. Rev. Cytol.* **1**, 135–164) has suggested. There is probably a close relationship between the factors conferring specificity in active transport and in immunological reactions. I have previously suggested that adaptive active transport arises by a process analogous to antibody formation (1954, *Symp. Soc. exp. Biol.*, in press). Pauling has shown that specific changes in proteins may be produced in artificial systems by small molecules. This indicates that the process postulated is feasible.

B. F. Folkes. Has Professor Danielli considered the energetic requirements of the uptake of KCl by cells? In plant roots, Robertson found that for an increase of oxygen uptake of one molecule, four molecules of KCl were taken up. Allowing for back diffusion this suggests that 1 high-energy phosphate bond is used for the uptake of one molecule of NaCl. Current theories suggest that many more ATP molecules are necessary for the contraction of one protein molecule. Does this not rule out the idea of contractile proteins in active transfer?

J. F. Danielli. No. The theory of active transfer of ions by protein contractions depends upon a protein oscillating between two alternative configurations. In the simplest case this will occur under thermal agitation, as in the dynamic equilibrium between native and denatured trypsin studied by Anson and Mirsy. In such instances the activation energy is low. If the activation energy for the change is high, thermal agitation may be supplemented by chemical energy derived from acetylcholine or ATP.

J. E. Harris. Are we confusing acetylcholine as a trigger with ATP as an energy source? The effect of acetylcholine on cilia is to produce a change in frequency of a contractile process the energy of which is derived from other sources. Whiting and I have found that acetylcholine has a similar effect on rhythmical contractions of embryonic voluntary muscles in the myogenic stage.

J. F. Danielli. I agree that acetylcholine may act upon a trigger mechanism in some instances of active transport.

L. M. Rinaldini. A single protein molecule might absorb a large number of ions and so transport many molecules of NaCl in a single contraction. In this case the 1 : 1 ratio between ATP and NaCl could still hold, but the number of molecules of ATP involved would provide enough energy to contract the protein molecule.

J. F. Danielli. I agree with this.

L. M. Rinaldini. Cinematography shows that cells are in a continuous state of flux, and that mitochondria move very actively within them, no doubt causing a stirring. The cell membrane and mitochondria are seen to stretch and contract very actively. Perhaps by constructing dynamic diffusion models some of the discrepancies between the figures obtained with models and with living cells might disappear.

J. F. Danielli. Studies on monolayers show that even with stirring there is a relatively unstirred layer close to the membrane. However the error introduced by this into calculations of permeability constants has been shown to be negligible unless the oil/water partition coefficient of the penetrating molecule is of the order of 1 or more. The molecular species we are considering here have lower coefficients.

If proteins are actively contracting on a protoplasmic surface they are likely to cause movement of that surface. This may indeed be the main method of protoplasmic movement, as has been suggested by Goldacre (1952, *Int. Rev. Cytol.* **1**, 135–164.)

K. E. Cooper. Does the absorption of red cell antibodies, or the agglutination of red cells by virus particles (e.g. influenza), or the treatment of red cells by enzymes (e.g. trypsin) affect permeability to ions?

J. F. Danielli. The patches on red cells specific for viruses are worthy of attention in this connexion, but I am not aware of any experiments bearing on this point. Prolonged treatment of red cells with proteases or lipases causes haemolysis, presumably owing to increased permeability to ions. The action of antibodies formed to known membrane components, such as cholinesterase, would be of great interest.

R. D. Keynes. When basic dyes are applied to the inside of the gill and fail to inhibit sodium transport, do they also fail to affect oxygen consumption?

H. Koch. We have not so far investigated this point.

J. F. Danielli. Pyocyanine has been shown to uncouple oxidation and phosphorylation. Is that true of the other dyes you used?

H. Koch. DNP has no influence on salt transport when applied to the outside of the gills. Therefore it seems unlikely that the effect of pyocyanine outside the gill is due to an uncoupling of oxidation and phosphorylation. Recently Judah and Ashman also described an interference of basic dyes with aerobic phosphorylation. It is certainly true that the interpretation of the action of basic dyes as anti-cholinesterases needs the support of more specific inhibitors of cholinesterases such as physostigmine, etc.

E. W. Yemm. At what pH were the experiments with 2–4 DNP carried out? Is this responsible for its action by influencing penetration?

H. Koch. Most of the experiments with DNP were carried out near pH 7, but those carried out at other pH values also failed to indicate any effect. When injected into the animal DNP proves to be an excellent inhibitor of active salt transport.

P. H. Tuft. Professor Koch says that the gills stain with basic dyes but that after washing activity reappears although the gills are still stained. Is it the chitinous cuticle which stains?

H. Koch. We observed that the dye which becomes free in very low concentration after the gill has been washed and replaced in 8 ml. NaCl is enough to inhibit activity again after a short time. This seems to indicate that certain definite points of the membrane must be occupied to obtain inhibition. This would fit in with the pore hypothesis.

R. J. Goldacre. A concentration of 10^{-3} M neutral red, which you found to inhibit active transport in *Eriocheir* gill, is near that which inhibits locomotion in *Amoeba proteus*. When locomotion stops in *Amoeba*, active transport of neutral red stops also. I was wondering whether the dyes in your experiments prevented contraction in the cytoplasm, which might explain some of your results.

H. Koch. Although the larger part of the gill membrane is covered with a chitinous cuticle, it is quite possible that dyes interfere with movements in the cytoplasmic membrane when reaching it at certain places.

Is it possible that neutral red interferes with the movement of *Amoeba* by acting on a cholinesterase in this animal?

R. J. Goldacre. I have tested acetylcholine on *Amoeba proteus* over a wide range of concentrations, and found no effect.

P. H. Tuft. Does the calcium-ion concentration have any effect on Na or K uptake by *Eriocheir* gills? It is said that the failure of this crab to invade Norwegian rivers is due to their low calcium concentration, and there is a connexion between calcium and acetylcholine.

H. Koch. The presence of calcium ions seems unnecessary for the active uptake of Na or K, and does not influence this uptake seriously, as far as I can judge from preliminary experiments. Perhaps Norwegian rivers also contain very little NaCl, and this may be a limiting factor. Low temperature may also have an unfavourable influence on the osmotic regulation and moulting of the crab.

Membrane structure as revealed by permeability studies

by

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It may be appropriate to take as the starting-point for the present discussion some experiments performed by Hevesy, Hofer and Krogh (1935) some twenty years ago. At that time Krogh was engaged in a study of the osmotic regulation of aquatic animals, and when heavy water (D_2O) became available it occurred to him that isotopic water might provide a valuable tool for characterizing the osmotic properties of biological membranes. In order to check the reliability of the new tool he wanted to perform determinations of the permeability of a living membrane to water with D_2O and, simultaneously, with the classical method of osmosis. As test material he chose frogs with a rubber bag sewn onto the cloaca to collect the urine formed during the experiment. The animals were submerged in tap water containing a suitable concentration of heavy water. Owing to the difference of osmotic pressure between the bathing solution and body fluids, water would be taken up osmotically. The uptake could be measured as the increase in weight of the frog plus the rubber bag. At the same time heavy water would exchange through the skin with ordinary water, as indicated by a drop in the deuterium concentration of the bathing solution.

In order to relate the exchange of heavy water to the net rate of uptake the authors made the very plausible assumption that the net uptake of water is equal to the difference between the amount of water diffusing in and that diffusing out.

The unidirectional diffusion of water may be taken, as a first approximation, to be proportional to the water concentration in the phase from which the diffusion takes place. If the bathing solution is pure water, its concentration is 55·5 moles per litre. The body fluid, however, which is some 0·2 osmolar with respect to solutes, is accordingly 55·3 molar with respect to water. Thus, for every 55·5 moles diffusing in, 55·3 moles will diffuse out, resulting in a net uptake of 0·2 moles.

The experimental results were not in agreement with these assumptions, however. The net uptake of water was between three and five times higher than the theoretical value calculated from the heavy-water flux and the difference in osmotic pressure across the skin. The authors concluded that until more became known, diffusion of heavy water could not be used to calculate rates of osmotic uptake.

In 1944 Visscher *et al.* made a study of the water movements between gut and blood of the dog, determining both the net water transfer and the rate of D_2O diffusion. Their theoretical assumptions were essentially the same as those of Hevesy *et al.* (l.c.), except that Visscher and collaborators assumed the rate of diffusion of water to be

proportional to the activity rather than to the concentration of the water. Even in this case the net transfers of water were much larger than predicted from the water activities, whether the gut contents were hypotonic or hypertonic with respect to the blood. Visscher took this as evidence that the water movements across the intestinal wall are due largely to active processes rather than to simple diffusion.

A few years ago in the Zoophysiological Laboratory of Copenhagen we resumed the study of water movements across the amphibian skin. The impetus to this study was a wish to clarify the mechanism underlying the so-called Brunn reaction or water balance reaction of anuran amphibians, which has been extensively studied in recent years (for references compare Heller, 1945, and Jørgensen, 1950). The reaction consists in an increased uptake of water through the skin following the injection into the animal of small doses of posterior lobe hormones. The response can also be elicited in the isolated skin of toads (Novelli, 1936) and frogs (Fuhrman and Ussing, 1951, Sawyer, 1951).

Since the Brunn reaction is more pronounced in toads than in frogs, skins of the former animal were used. An apparatus was designed which allowed the determination of the net water-transfer rates with an accuracy of $\pm$ 10 μl. and, simultaneously, the measurement of the water-diffusion rate, using 5 per cent. heavy water as a tracer. As inside medium ordinary Ringer solution was used, whereas the outside medium was 1/10 Ringer.

Some typical results are shown in Table I (Koefoed-Johnsen and Ussing, 1952). The heavy-water diffusion figures are calculated as total influx values (M_{in}) expressed as the amount that would pass through unit area in unit time if the heavy-water concentration were maintained at 100 per cent. in the outside compartment and at zero in the inside compartment. The net water flux, Δ_w, as well as the influx, is given in μl./cm.^{2}/hr.

The results confirm in every respect those of Hevesy, Hofer and Krogh (l.c.) on live frogs. For the sake of argument, let us assume that the water uptake is due to simple osmosis and that the net uptake is the difference between two diffusion streams. The permeability coefficient as calculated from heavy-water diffusion, namely P_{diff}, is defined by the equation

$$M_{in} = P_{diff}\, c_{w(o)}$$

For $M_{in} = 532$ μl./hr. P_{diff} works out to be $1{\cdot}48 \times 10^{-4}$ cm./sec. In the same experiment Δ_w was 30 μl./hr.

Now, for Δ_w we have

$$\Delta_w = M_{in} - M_{out} = P_{osm} c_{w(o)} - P_{osm} c_{w(i)} = P_{osm}(c_{w(o)} - c_{w(i)})$$

Remembering that Δ_w and $(c_{w(o)} - c_{w(i)})$ should be expressed in the same units, we get

$$P_{osm} = 2{\cdot}32 \times 10^{-3}$$

or nearly 16 times the figure for P_{diff}.

It is seen that the influx changes only slightly on the addition to the inside solution of posterior lobe extract. The flux may even go down. But the net flux always increases violently, often by more than 100 per cent. In the beginning we took this finding as an indication that the hormone evokes an active transport of water, a view

which was also taken by Capraro *et al.* (1952) who obtained similar results with isolated frog skins. There was, however, something mysterious about this apparant active transport. It could take place only if there was an osmotic gradient to help it. With isotonic sucrose in the outside compartment and Ringer solution inside, the net water transfer was nil. This is in keeping with the observation made by Krogh years ago that frogs placed in isotonic sucrose will not take up water and do not form

TABLE I

Effect of neurohypophysial hormone on influx and net flux of water through toad skin. Inside solution, Ringer solution; outside solution, 1/10 *Ringer (Koefoed-Johnsen and Ussing,* 1952)

M_{in} = influx of water (μl./cm.^{2}/hr.)

Δ_w = net flux of water (μl./cm.^{2}/hr.)

Date		Control periods				1 hr. and 2 hr. periods after addition of neurohypophysial powder			
		a		*b*		*c*		*d*	
		M_{in}	Δ_w	M_{in}	Δ_w	M_{in}	Δ_w	M_{in}	Δ_w
24/1	M_{in}	441		460		532		551	
	Δ_w		12·0		13·0		30·0		36·0
28/1	M_{in}	305		319		292		310	
	Δ_w		6·7		5·0		10·8		7·7
30/1	M_{in}	343		370		334		404	
	Δ_w		9·7		7·4		16·0		17·0
31/1	M_{in}	326		287		344		369	
	Δ_w		11·7		8·0		21·0		25·0

any urine. It is true that the isolated skin with Ringer solution on both sides performs a transfer of water from the outside medium to that inside (Huf, 1936), but this is probably connected with the active transport of sodium ions through the skin.

The fact that the apparently active water transport needs an osmotic gradient to help it started us wondering whether or not our basic concepts of the nature of osmotic water transfer were correct. Does the net water transfer indeed arise as the difference between the amount of water diffusing in and that diffusing out? At first sight even the asking of the question seemed to us preposterous, but on second thoughts we realized that the problem needed reconsideration.

In order to make the point clear, let us consider a model system which, in an exaggerated form, illustrates the problem. The system consists of two compartments, *I* and *O*, which are separated by a 'membrane'. The 'membrane' is largely impermeable, communication of solvent between *I* and *O* being possible only through a number of pores which have the shape of small osmometers with the semi-permeable membrane facing towards *I* and the long narrow stem opening into *O*. Compartment *I* contains sucrose dissolved in heavy water, whereas the outside medium is pure ordinary water. Owing to the osmotic effect of the sucrose, water will be sucked through the semi-permeable membrane and water will be replenished via the stems of the osmometers. If the area of the semi-permeable membrane is large and the diameter of the stem is small, the linear rate of water flow in the stems may easily exceed the diffusion rate of water. Consequently, although water can easily pass from *O* to *I*, the heavy water of the inside solution can never reach *O* although it passes easily enough through the semi-permeable membrane. This model system, as already mentioned, represents an exaggerated picture of something that will always occur in pore membranes.

Let us now consider a simple pore membrane which is impermeable, for instance, to sucrose. At the boundary of the membrane adjoining the sugar solution events are governed by the ideal law. The net flux arises as the difference between the water diffusing out of the sugar solution and that diffusing into it, and we can write

$$M_{in}/M_{out} = a_{w(o)}/a_{w(i)};$$

but, since the water phase filling the pores is pure water, it will only flow to replenish that lost by osmotic suction in so far as a difference of hydrostatic pressure is built up between the ends of the pore. In other words, that part of the water transfer process concerned in overcoming the internal friction in the membrane phase is governed by the laws of laminar flow and not by the laws of diffusion. Now these laws are of a very different nature. For a pore of given length the amount of water which can diffuse through in a given time under steady-state conditions depends on the area, or in other words, on the radius to the second power. Laminar streaming through a cylindrical pore, according to Poiseuille's law, is proportional to the radius to the fourth power. We can put this a little differently and say that for a given area diffusion is independent of the number of pores in which this area is divided up, whereas the flow of water is proportional to the second power of the pore radius.

It is quite easy to express these considerations in mathematical terms. I shall not take your time by developing the expressions, but shall confine myself to presenting a few of the resulting expressions. It turns out that the following expression is generally valid for a semi-permeable membrane:

$$\ln \frac{M_{in}}{M_{out}} = \frac{\Delta_w}{D_w} \int_0^{x_o} \frac{1}{A} dx \quad . \quad . \quad . \quad . \quad (1)*$$

* *Footnote* : $\ln \frac{M_{in}}{M_{out}}$, indicating the 'one-sidedness' of the process, has the dimension of a potential. Δ_w is a 'current strength', whereas $\frac{1}{D_w} \int_0^{x_o} \frac{1}{A} dx$ is the diffusion 'resistance'. Thus the whole expression is analogous to Ohm's law.

The meanings of M_{in}, M_{out} and Δ_w have been defined above. D_w is the diffusion coefficient for water diffusing in water, A is the fraction of the total area of the membrane which is available to water diffusion, x is the distance from the inside boundary, and x_0 is the total thickness of the membrane.

Evidently the flux ratio for water may vary profoundly, and depends on the shape of the pores inside the membrane.

In the case of the action of posterior lobe on the toad skin, in which the net water flux increased by more than 100 per cent. without the influx's changing by more than a few per cent., it turns out that the equation is satisfied if the diffusion area remains constant while, at the same time, a larger number of narrow pores is replaced by a smaller number of large pores. The results therefore do not necessarily indicate an active transport of water. But the alternative to the active transport hypothesis is the acceptance of pores in the membrane. In order to see what the pore hypothesis means in terms of pore dimensions it may be useful to consider an 'equivalent' membrane with uniform cylindrical pores. Furthermore it is assumed that the only force available for the transfer of water is the difference of osmotic pressure across the membrane. We then get the following simple expression:

$$M_{in}/M_{out} = \left(\frac{a_{w(o)}}{a_{w(i)}}\right)^{G_w/g'_w} \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where G_w is the frictional coefficient for water diffusing in water, and is equal to RT/D_w. D_w has been determined by Orr & Butler (1935) and more precisely by Rögener (1941). At 17·5° C. the numerical value of G_w is $1{\cdot}36 \times 10^{15}$. The term g'_w represents the frictional coefficient for water flowing through the membrane. It is a function of the pore diameter, and works out as

$$g'_w = \frac{144\eta}{r^2}$$

where η is the viscosity of water. At 17·5° C. we have

$$g'_w = \frac{1{\cdot}5}{r^2}.$$

It is seen that G_w becomes equal to g'_w for $r = 3{\cdot}5 \times 10^{-8}$ cm. Since this is less than the average distance between water molecules, we must conclude that at all real pore sizes *water flow takes place with a lower resistance than water diffusion*. As one might expect, the difference between the two frictional coefficients vanishes when one gets down to molecular dimensions, and one obtains the classical equation as applied by Hevesy *et al.* (l.c.) and Visscher *et al.* (l.c.). With increasing pore size, however, the frictional resistance for flow gradually becomes insignificant as compared with that for diffusion.

Inserting the numerical values for G_w and g'_w in equation (2), we obtain:

$$\log (M_{in}/M_{out}) = 0{\cdot}9 \times 10^{15} r^2 \times \log(a_{w(o)}/a_{w(i)}) \quad . \quad . \quad . \quad (3)$$

In one of the toad-skin experiments mentioned above, after the hormone had been added, the water influx was 532 μl./hr. and the net flux 30 μl. Taking the water activities to be equal to the water concentrations we had

$$c_{w(i)} = 55{\cdot}3 \text{ mol./l. and } c_{w(o)} = 55{\cdot}5 \text{ mol./l.}$$

With these figures inserted in equation (3) the pore radius, r, works out as $13{\cdot}4 \times 10^{-8}$ cm. The same skin before the hormone treatment gave an equivalent pore diameter of $9{\cdot}25 \times 10^{-8}$ cm.

Table II shows the molecular dimensions of a number of biologically interesting substances, collected and in part also determined by Pappenheimer (1953). Thus

TABLE II

Dimensions of some biologically interesting molecules (after Pappenheimer, 1953)

Molecular species	Molecular dimensions in cm. $\times 10^{-8}$		
	Radius of equivalent sphere		Dimensions estimated from X-ray diffraction or from frictional ratios
	From free diffusion	From intrinsic viscosity	
NaCl	1·4	—	Cl^-, radius 1·8 Na, radius 0·95
Urea	1·6	—	—
Glucose	3·6	3·8	5 × 7 × 9
Sucrose	4·4	4·4	8 × 11 × 12
Raffinose	5·6	5·7	axes of equiv. ellipse $a = 16$ $b = 10$
Inulin	15·2	15·3	axes of equiv. ellipse $a = 96$ $b = 22$
Muscle haemoglobin	19·0	—	cylinder, $d = 54$ $h = 8$
Haemoglobin	31·0	35·0	cylinder, $d = 54$ $h = 32$

the frog-skin equivalent membrane has a pore size which is smaller than the diameter of the inulin molecule but larger than the molecules of most crystalloids. However, as pointed out by Pappenheimer in his very important paper, there is a considerable restriction to diffusion of molecules only slightly smaller than the pore diameter. These calculations show that the concept of diffusion and flow through

pores does not lead to inherently nonsensical results. But after all, you may say, the amphibian skin is a multicellular structure and the pores might be in the intercellular substance rather than in the cell membranes proper. Prescott and Zeuthen (1952) have, however, determined the diffusion permeability and the osmotic permeability of eggs of various freshwater animals and have found the two to be distinctly different. Some examples are shown in Table III. It will be seen that, particularly in the ovarian eggs, the two permeability coefficients differ appreciably. We can therefore say with some certainty that these cells have pores in the membrane, or else, perhaps, something that experimentally shows up as pores. What I mean with this is that they need not be permanent structures, but may form and close continuously. This possibility cannot, however, be tested with our present techniques.

TABLE III

Permeability to water of various eggs (after Prescott and Zeuthen)

Diffusion permeability coefficient: P_d.

Filtration permeability coefficient: P_f.

Cell type	P_d μ/sec.	P_f μ/sec.	P_f/P_d
Frog ovarian egg	1·28	89·1	69·0
Zebra fish ovarian egg	0·68	29·3	43·0
Xenopus body-cavity egg	0·90	1·59	1·8
Frog body-cavity egg	0·75	1·30	1·7
Zebra fish shed, non-developing	0·36	0·45	1·3

It would be very interesting to obtain similar information concerning other cell types. The technique of Prescott and Zeuthen (l.c.), making use of the diver balance to determine the D_2O exchange rate as well as volume changes, lends itself to the study of the permeability to water of many large and medium-sized cell types.

The idea that living membranes have pores is of course not a new one. Thus Collander's (1937) well-known lipoid-pore theory implies that small hydrophilic molecules penetrate living membranes largely through pores. Davson and Danielli (1943), however, have pointed out that the evidence given by Collander for his theory is not quite conclusive. The existence of two different permeability constants for water in many membranes can be taken as support for the lipoid-pore theory.

If pores in living membranes do exist, this has certain obvious consequences with respect to the way in which the flow of water through the pores influences the diffusion of dissolved substances. Molecules which diffuse upstream will be slowed down, whereas those diffusing downstream will be speeded up. Such 'drag effects' may be of importance in processes of secretion and in apparently 'active' transport. This effect will be greater for big molecules than for small ones, because the contribution to the over-all movement will be less for slow molecules. On the other hand,

the larger molecules may not be able to penetrate the pores at all. In order to measure the effect one ought therefore to use molecules as large as possible among those which do penetrate. This imposes the experimental difficulty that the concentration changes in the solutions in contact with the membrane are likely to be immeasurably small.

But here the double-labelling tracer technique may prove useful. In order to study the water drag effect we have prepared (Andersen and Ussing, in preparation) thiourea labelled with ^{14}C which, together with the commercially available ^{35}S-labelled thiourea, gives a suitable pair. Thiourea is very water-soluble and hardly soluble in lipoids. If pores are present it is therefore likely to follow these. Another interesting feature is that since it is much larger than the water molecule it is likely to penetrate mostly through the larger pores where the linear rate of water flow and thus the drag effect is larger.

The experimental approach is the following: A toad skin is placed as a membrane with Ringer solution on the inside and 1/10 Ringer on the outside. ^{14}C-labelled thiourea (20 mg. per cent.) is added to the outside solution and ^{35}S-thiourea of equal concentration is added to the inside. It is then possible to measure both influx and outflux of the substance although the transfers are far too small to be measured chemically. The first experiments of this type were completed only a few days ago. It is therefore only possible to give a few examples. In one experiment the influx and the outflux were both $7{\cdot}9 \times 10^{-10}$ mol/cm^2/hr. Then posterior lobe hormone (1 unit per 20 ml.) was given to the inside solution. In the following three hours the influx rose to $56{\cdot}8 \times 10^{-10}$ whereas the outflux rose relatively less, to $43{\cdot}1 \times 10^{-10}$. Thus the flux ratio ($M_{in}/M_{out}$) was 1·00 in the first period but 1·31 during the period of the hormonally stimulated water flow.

Experiments of this type have, of course, to be performed under varying conditions and also with various test substances. We hope, however, that it will prove possible by this approach to obtain quantitative measures of some properties of living membranes which have been hitherto very difficult to obtain.

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Discussion

Chairman: J. Brachet

R. D. Keynes. Are you inclined to doubt the existence of mechanisms for the active transfer of water in other tissues? There seems to be good evidence for the active absorption of water in the intestine, for example.

H. Ussing. I am quite convinced that active transport of water does occur in certain organs. But I want to emphasize that a discrepancy between the water activity ratio and the water flux ratio may be an indication of the presence of pores rather than an indication of active water transport. It is curious that, when isotonic sucrose is placed outside, there is no transfer of water across frog skin.

H. Heller. Would you conclude that the effect of neurohypophysial hormone is on pore size, and if so how is it produced?

H. Ussing. Yes, but the experimental evidence does not indicate the means.

J. F. Danielli. To what extent would the occurrence of pinocytosis modify your analysis?

H. Ussing. The analysis I have used is a purely formal one, and in certain cases pinocytosis might simulate a porous membrane. Pinocytosis is likely, however, to influence all solutes in the same way. It is our hope that, by performing the analysis with a series of substances, certain concepts may be ruled out and others become more likely.

E. Zeuthen. Pinocytosis should be unidirectional, but swelling and shrinkage indicate a capacity of water to pass both ways.

J. F. Danielli. Some tissue culture cells are continually taking up water by pinocytosis. It must leave the cell again, but how?

J. A. Kitching. The cells of *Hydra* are permeable to water, and the internal osmotic pressure exceeds the external, so that the same problem arises here.

R. J. Goldacre. I understand that the area occupied by the pores would be only a small fraction of the total area of the membrane. In the frog skin, could the spaces between the cells be adequate to account for the effect observed, or do you require pores also in the plasma membrane of each cell?

H. Ussing. Neurohypophysial hormones decrease the resistance to active transport of Na as well as the resistance to water flow. It therefore seems that water and Na should follow in part the same paths. If, as seems likely, the active transport of Na goes through the cell membrane, the latter would seem to have pores. Moreover,

during active transport of Na the outflux is only a minute fraction of the influx. If intercellular spaces were important, the outflux would be considerable.

J. F. Danielli. It is suggested that the surface of *Beggiatoa mirabilis* has pores of a diameter slightly less than that of the sucrose molecule.

W. G. B. Casselman. Recalling the influence of the neurohypophysial hormone on renal tubercules, have your experiments on frog skin provided any evidence of differential changes of permeability?

H. Ussing. Neurohypophysial hormone increases the active transport of sodium ions very appreciably, apparently by lowering the resistance to this ion. On the other hand permeability to the chloride ion is only slightly affected.

The ionic permeability of nerve membranes

by

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One of the most striking characteristics of living cells is the existence of large ionic concentration gradients across the membranes which bound them. In studying the ionic permeability of cell membranes, we have to investigate not only the active transport mechanisms by which the concentration gradients are built up in the first place, and the properties of the membrane on which their maintenance depends, but also the important question of the part played by ionic permeability in fulfilment of the normal biological function of the cell. In many cases an active transport system is necessary to maintain an osmotic balance between the intracellular and extracellular fluids. In others a high internal concentration of certain ions may be advantageous, or even essential, for the optimal working of enzyme systems. Some cells form part of a secretory organ, and are capable of transferring, often against considerable concentration gradients, large amounts of the particular ions for whose transport they are adapted. In the example with which I am concerned, a rather different adaptation has occurred, the ionic concentration gradients being utilized, through special behaviour of the cell membrane, to form a system which can conduct a transient reversal of membrane polarization rapidly from one end of the cell to the other. I will consider first the role of ions in the passage of a nerve impulse, as it is from this aspect of the permeability problem that we are likely to observe the greatest specialization of the membrane. I will then turn to some evidence on the recovery process in giant axons, where it would not be unreasonable for the mechanisms at work to be less highly differentiated, and possibly similar to those in other types of cell.

The story begins with the discovery by Hodgkin and Huxley (1939, 1945) and Curtis and Cole (1942) that the action potential in a nerve fibre does not consist simply in a depolarization towards zero membrane potential, as Bernstein (1912) had supposed, but involves a temporary reversal of potential by some 40 mV. Since these pioneer experiments on giant squid axons, the introduction of methods for measuring membrane potentials by means of 0·5 μ glass microelectrodes thrust into the interior of cells (Ling and Gerard, 1949; Nastuk and Hodgkin, 1950) has yielded reasonably reliable values for the absolute sizes of the potentials in a wide variety of excitable tissues. Recent additions to the list given by Hodgkin (1951) are the studies of Brock, Coombs and Eccles (1952) on mammalian motoneurones, and of Keynes and Martins-Ferreira (1953) on the electroplates of the electric eel. It is noteworthy that although the duration of the action potential may vary from less than one millisecond to several hundred milliseconds, the sizes of the membrane potentials cover a

very much narrower range. The resting potential generally lies between 60 and 90 mV, while during activity the potential is reversed by 30 to 60 mV.

Another feature which all these tissues have in common is their possession of a high internal potassium and low sodium content. The actual concentrations are, of course, higher in marine invertebrates like squid and cuttlefish (the body fluids of which are isotonic with sea water) than in mammals and other vertebrates, but there is a general similarity between the concentration ratios in all species. Thus there is usually about twenty times as much potassium inside the cells as outside, but only one-tenth as much sodium. This has been shown particularly well in the case of giant squid axons, the axoplasm of which can be extruded and analysed without any complications arising from the presence of indeterminate quantities of extracellular material.

These observations can most satisfactorily be explained on the basis of the ionic hypothesis put forward by Hodgkin, Huxley and Katz, the evidence for which has been reviewed by Hodgkin (1951). It is suggested that the resting nerve membrane is relatively permeable to K^+ and Cl^- ions, and impermeable to Na^+ ions. When the membrane is depolarized by 15 mV or more, either by application of a cathode, or by local circuit action when a neighbouring portion of the nerve becomes active, its permeability to Na^+ rises temporarily much above that to any of the other ions present. Sodium ions then begin to move inwards, driven by the concentration gradient, thus depolarizing the membrane further, and increasing the sodium permeability still more in a regenerative fashion. The inward movement of sodium continues until the peak of the action potential is reached. Here it ceases, both because the mechanism responsible for raising the sodium permeability becomes inactivated, and because the membrane potential has now arrived at a level close to the equilibrium potential for sodium. At this point, the potassium permeability of the membrane is raised to a value considerably greater than its resting one, and a net outward movement of K^+ ions takes place, quickly restoring the membrane potential to its original resting level. After a brief refractory period while the sodium and potassium permeability systems recover to their normal quiescent state, the nerve is ready to conduct another impulse. It has lost a small amount of potassium in exchange for sodium, and it is from these downhill ionic movements, which must ultimately be reversed by an ionic pump harnessed to metabolism, that energy is derived for the electric currents which flow during propagation of the impulse.

The major pieces of evidence in support of these ideas are as follows:

(1) Conduction is blocked in a medium from which sodium is absent. Exceptions to this statement are that lithium, but no other cation, will act as a substitute for sodium, and that in crustacean muscle the mechanism of conduction appears to differ from that just described (Fatt and Katz, 1953).

(2) The relationship between external sodium concentration and the extent to which the membrane potential is reversed at the peak of the spike conforms closely to that predicted by the hypothesis. Desmedt (1953) has now shown that in frog muscle the effect of varying the internal sodium concentration also fits well with theoretical expectation.

(3) Studies with radioactive sodium and potassium have shown that in non-myelinated invertebrate nerves the effect of stimulation is to accelerate the ionic movements in both directions. From these experiments, and from analyses of squid

and *Sepia* axons, it has been found that during activity there is a net gain of sodium and a roughly equal net loss of potassium which is more than large enough to account for the changes in membrane potential.

(4) The laws governing the movements of sodium and potassium during activity have been studied in squid axons by Hodgkin, Huxley and Katz (1952), using a technique by which the flow of current through a fixed area of nerve membrane was measured while the membrane potential was varied in a strictly controlled manner by a feed-back amplifier system. Comparison of results in normal and in sodium-free sea water (choline being substituted for sodium) enabled the separate contributions of Na^+ and K^+ ions to the total ionic current to be evaluated, and provided strong evidence that the sodium permeability of the membrane rises to a maximum soon after the initiation of an impulse and is subsequently inactivated, while the potassium permeability only builds up after an appreciable delay. From a detailed analysis of their results, Hodgkin and Huxley (1952) were able to show that such a sequence of permeability changes could account quantitatively as well as qualitatively for various well-known features of conduction and excitation.

(5) The effects of varying the external sodium and potassium concentrations have shown that in myelinated vertebrate nerve the active changes in membrane potential probably involve mechanisms similar to those in non-myelinated nerve. But the excitable membrane is confined to a restricted area at each node of Ranvier, the insulated internodal stretches of the fibre behaving as purely passive conductors.

All the ionic movements I have described so far could occur without the intervention of metabolism, since in each case they involve the transfer of ions from a strong to a weaker solution. There is, however, ample evidence that metabolism does play an essential part in the continued functioning of peripheral nerves. It has often been shown, for example, that nerves deprived of oxygen will sooner or later cease to conduct impulses, and that they will recover on the readmission of oxygen (see Shanes, 1951). In a similar way, transmission through a mammalian sympathetic ganglion is dependent on an adequate supply both of oxygen and of glucose (Larrabee and Bronk, 1951). It is also well known from the work of A. V. Hill and his collaborators (see the review by Feng, 1936) that there is a rise in heat production during nervous activity, and there has recently been a renewed interest in the increase in oxygen consumption of stimulated nerves (Brink, Bronk, Carlson and Connelly, 1952), and in their carbon dioxide production, which varies according to the substrate metabolized (Mullins, 1953). We must next consider the rather meagre evidence as to the precise relation between nerve function and nerve metabolism.

Shanes (1951) has described an experiment on a partially cleaned squid axon which was mounted in a moist chamber and exposed to pure nitrogen. After about thirty minutes of asphyxia, conduction failed, but the block probably arose from an accumulation of potassium in the thin layer of external fluid, since it could be relieved at once (though not for more than a few minutes) by flushing the apparatus with nitrogenated sea water; a return to an atmosphere of oxygen also restored conduction, but only with a lag of some minutes. Hodgkin and I (1954*b*) have done a similar experiment on a *Sepia* axon mounted in oil, in which we found that a normal axon was able to maintain a steady state during stimulation at a low rate by reabsorbing potassium as fast as it leaked out, while poisoning it with dinitrophenol

prevented potassium absorption, and soon made it inexcitable. Excitability could again be restored, immediately but not for long, by washing the outside of the axon in fresh sea water still containing dinitrophenol. These observations suggest that, at least in cephalopod axons, the primary function of nerve metabolism is to provide energy for the recovery processes which are responsible for the absorption of potassium and extrusion of sodium after activity, and that energy-yielding metabolic mechanisms do not intervene directly in the generation of the action potential.

The problem has been studied in more detail with the help of radioactive isotopes, and with intracellular microelectrodes. In squid and *Sepia* axons loaded with ^{24}Na there is a continual outward movement of the isotope through the cell membrane, which apparently results from the operation of an active transport mechanism. Blocking of metabolism with dinitrophenol, cyanide or azide, results in a gradual reduction of the sodium efflux to about one-twentieth of its initial value, and the efflux can later be restored by washing the inhibitor away (Hodgkin and Keynes, 1953*a*, 1954*a*). This inhibition of the sodium pump has been observed under a wide variety of experimental conditions; it occurs whatever method is used to introduce ^{24}Na into the axon, and is very little affected by changes in the external medium—the effect even persists in an axon soaked in an isotonic dextrose solution containing almost no salts. Somewhat to our surprise, we have also found that the potassium influx is cut down by inhibitors to about one-seventh of its resting value. This conflicts with the earlier view (see Keynes, 1951) that the fluxes of K^+ ions moving across the membrane are wholly passive, but fits with other recent evidence suggesting that the active transport mechanism works by an inward potassium transfer more or less tightly coupled to the sodium extrusion. Thus in cephalopod axons (Hodgkin and Keynes, 1953*b*) abolition of the potassium influx by removing all the external potassium results in a reversible decrease of the sodium efflux. The interaction of sodium and potassium fluxes cannot be mediated through the usual effect of potassium concentration on the resting membrane potential, since we have found (Hodgkin and Keynes, 1954*a*) that the sodium efflux in *Sepia* axons is not altered perceptibly by quite large polarizations of the membrane, so that there must be some more specific form of coupling between them. It is tempting to suggest that such coupled ionic pumps may be quite widespread, although the only supporting evidence available at present is that a similar effect of external potassium on sodium efflux has been observed both in erythrocytes (Harris and Maizels, 1951) and in frog muscle (Keynes, 1954).

This type of coupled pump would be neutral in that it would transfer no net charge across the membrane. The evidence just considered is therefore consistent with the further observation that in a squid axon, poisoning with dinitrophenol only causes a slow decline in the resting and action potentials (Hodgkin and Keynes, 1954*a*), as would be expected if under these conditions the intracellular potassium content is falling, and sodium is rising, faster than in an untreated axon. We have also confirmed with ^{24}Na that the rapid sodium movements during activity are almost unaltered by dinitrophenol, when at the same time the resting sodium efflux has been brought to a standstill. In cephalopod axons it seems clear that there can be no very direct connexion between the mechanisms involved in conduction and in recovery, since each can go on working when the other is put out of action (an

axon depolarized by a high external potassium concentration cannot conduct impulses, but continues to extrude sodium). It does not follow, however, that this is necessarily true for other tissues. A sodium pump which extruded a stream of Na^+ ions, like the system in frog skin examined in Ussing's elegant experiments (see Ussing and Zerahn, 1951), would make a definite contribution to the resting potential, and such a pump may well be present in mammalian muscle and nerve. There is, indeed, a suggestion of the sort in the recent paper by Bennett, Ware, Dunn and McIntyre (1953) on the resting potential in mouse muscle fibres *in vivo*, some of their values being higher than any reasonably attributable to a potassium-diffusion potential.

It must be appreciated, too, that giant cephalopod axons have an abnormally large ratio of volume to surface, and are hence enabled by their ionic reserves to conduct hundreds of thousands of impulses before any recovery is essential. The situation may be similar in myelinated nerves, in view of their greatly reduced area of active membrane, but is likely to be different in nerve cells having numerous fine dendrites, where the ionic reserves may in effect suffice only for the conduction of a few impulses before they need to be recharged. The marked dependence of the cells of the mammalian central nervous system on a continuous supply of glucose and oxygen is thus not surprising, whether or not they work in precisely the way I have described for non-myelinated invertebrate nerves.

Two extremely interesting questions about which we are still wholly ignorant are those of the chemical identity of the sodium and potassium carriers, and of the nature of the coupling between them and cellular metabolism. Very few chemical compounds are known to be able to discriminate between sodium and potassium as efficiently as the cell membrane, and there is no evidence that any of them are actually found in living cells. The obvious suggestion to make about the link with metabolism is that the sodium pump derives its energy from ATP. This would fit with the facts that in cephalopod axons, which probably have only a small reserve of energy-rich phosphate bonds, the sodium extrusion ceases quite rapidly on interference with metabolism, whereas in frog muscle, which is rich in phosphocreatine, metabolic inhibitors have no very obvious effect on the sodium efflux (Keynes and Maisel, 1954). Moreover Nachmansohn, Coates, Rothenberg and Brown (1946) have presented evidence that ATP and phosphocreatine participate at some point in the discharge of the electric organ. But there is no compelling proof that ATP plays a direct role in driving active transport systems, and we should not ignore the possibility that the fuel consumed by the sodium pump is really some other end-product of metabolism.

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Discussion

Chairman: J. Brachet

N. Myant. What ionic movements occur across the membrane between the nodes in a myelinated mammalian nerve?

R. D. Keynes. The experiments of Huxley and Staempfli (1949: *J. Physiol.* **108**, 315–339) showed that there was only a small outward current, probably carried by the K^+ ions, through the myelin sheath. In contrast to the larger currents flowing in and out at the nodes, this could be explained as a purely passive current due to the potential change acting on a resistance and capacity in parallel.

R. J. Goldacre. Has any attempt been made to follow visually the course of active transport of ions in nerve by the use of cationic dyes? Although the emphasis is on the specificity of these pumps, it is difficult to think that a dye like neutral red would not be taken up by nerve to an extent which would perhaps be sufficient, in the case of a giant axon, for its course to be followed under the microscope.

R. D. Keynes. We have never seriously investigated the penetration of dyes into giant axons. Dyes injected into giant axons seem to diffuse as far as the membrane and no further.

J. E. Harris. Is there any connexion between the phenomena you have just described and the very active uptake by nerves of methylene blue?

R. D. Keynes. I do not know of any physico-chemical connexion between the activity in a nerve and the uptake of methylene blue; but I suppose that it is conceivable that the dye might enter at the nerve terminals during the non-specific increase in permeability which is thought to occur as a result of liberation of acetylcholine.

Cellular oxidations and the synthesis of amino-acids and amides in plants

by

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INTRODUCTION

THE biochemical mechanisms engaged in the synthesis of amino-acids and proteins in the cell have recently been extensively studied. There is now much evidence that amino-acids are directly involved in the biosynthesis of proteins, but two distinct hypotheses have been put forward with regard to the way in which specific peptide structures are built up. The first of these, the so-called 'template' hypothesis, was advanced primarily to account for the reduplication of protein structures; it suggests that amino-acids are orientated on specific surfaces in the cell and are there condensed *en bloc* in a single-step reaction. In a review of this mechanism, Dounce (1952) considers that transphosphorylations, mediated by nucleic acids, may provide the energy coupling necessary to promote the reaction. The second hypothesis, developed mainly by Fruton (1952) and Waelsch (1952), suggests that a preliminary synthesis of amino-acid amides or simple peptides takes place, followed by a conversion to proteins by transamidation and transpeptidation reactions controlled by specific transferring enzymes in the cell. As distinct from the 'template' hypothesis, this transamidation mechanism implies an active formation of amides and simple peptides and a close coupling between these syntheses and the exergonic reactions of cell respiration. It is the main objective of this paper to consider some further evidence, which has recently been obtained, bearing on this point. An attempt has been made to trace some of the stages by which simple inorganic forms of nitrogen are assimilated by plant cells. Under favourable conditions a rapid formation of amino-acids and amides from ammonium salts or nitrates takes place, and affords an opportunity of examining the relation between these syntheses and the breakdown of carbohydrates in cellular oxidations.

CELLULAR RESPIRATION AND THE ASSIMILATION OF NITROGEN

It is well established that the rate of respiration of plants and micro-organisms may be greatly increased during the assimilation of nitrogen. Kellner (1874) first showed that pea seedlings respired more rapidly when supplied with nitrates, and his observations have been confirmed and extended to other species by Hamner (1936), Hoagland (1944), Woodford and Gregory (1948), Humphries (1951), and Syrett (1953). Our work in this direction has been carried out mainly with young seedlings

of barley and with food yeast, *Torulopsis utilis*. These materials were chosen because, despite wide differences in general nutrition, they both have a high capacity for assimilating nitrogen and synthesizing proteins from simple inorganic compounds of nitrogen. For example, cultures of food yeast, supplied with ammonium salts under favourable conditions, will double their protein content within 2–3 hours. High rates of assimilation and protein synthesis also obtain in the early stages of development of barley seedlings. The conditions which favour a rapid uptake of ammonium salts

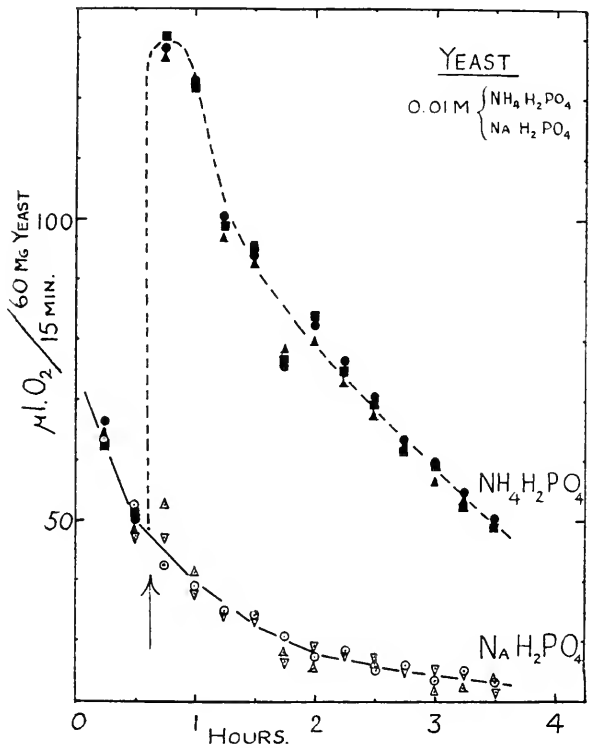


Figure 1. *The effect of ammonium ions on the rate of oxygen uptake of yeast. Replicate samples, containing* 60 *mg. of fresh yeast, were treated at the time indicated with either ammonium or sodium phosphate. Oxygen uptake was measured by conventional manometric methods.*

or nitrate were investigated in a series of preliminary experiments and an account of these has already been given (Folkes, Willis and Yemm, 1952; Yemm and Folkes, 1954). The chief results may be briefly recorded.

An essential condition for rapid assimilation is a high level of readily available carbohydrates in the cells. With both organisms this requirement can be met by growing them for a short preliminary period under conditions of high carbohydrate supply, but deprived of nitrogen. The seedlings were therefore grown at high light intensities in a nutrient solution deficient in nitrogen. A similar effect is achieved

with yeast by treating the cultures in aerated solutions containing sugars and other mineral nutrients but no nitrogen. In this way a high level of soluble sugars or polysaccharides is built up in the cells, and their ability to assimilate nitrogen in the absence of external supplies of carbohydrate is greatly increased. A further point, shown by the experiments with seedlings, was that the primary reactions, associated with the assimilation of nitrates or ammonia, occur mainly in the root system. On this account most of the work considered here has been carried out with roots immediately after their excision from the growing seedling.

Experiments both with yeast and root tissues have shown consistently that the

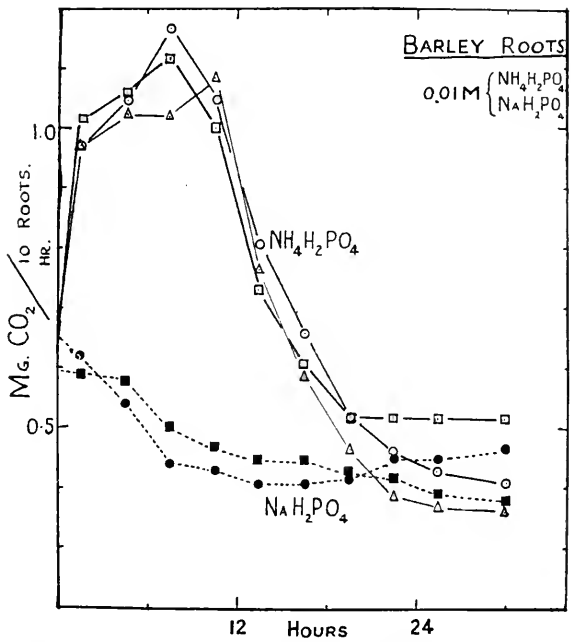


Figure 2. The effect of ammonium phosphate on carbon dioxide production of excised barley roots. Samples of 40 root systems, freshly cut from the seedlings, were treated in aerated culture solutions with ammonium or sodium phosphate. CO_2 output was measured by the Pettenkofer method.

rate of oxygen uptake increases rapidly when ammonium salts are supplied under favourable conditions. The rate of carbon dioxide production or oxygen consumption is commonly more than doubled within a short time of supplying the ammonia, as illustrated by typical results in Figures 1 and 2. The highest rates of respiration are maintained for only a short time and it is very probable that depletion of the limited carbohydrate reserves in the cells is an important factor causing the secondary decline in rate. No external supply of sugar was provided in these experiments, and analytical data, which are considered in a later section, show that a rapid breakdown of carbohydrates is associated with intense respiratory activities during the assimilation of nitrogen.

It may be noted that nitrates, nitrites and, to a less extent, hydroxylamine increase the rate of respiration in barley roots. But here there is evidence of greater complexity compared with the effects of ammonium salts. The respiratory quotient rises considerably above unity with nitrates and nitrites, suggesting that they act as hydrogen acceptors in the oxidation mechanism. The action of hydroxylamine is complicated by its toxic effects even at low concentrations.

An account of the experiments with barley roots has been given by Willis (1950, 1951).

THE PRODUCTS OF NITROGEN ASSIMILATION

In an attempt to identify some of the reactions associated with the high rates of cellular oxidation, the products formed in the cells during the early phases of assimilation have been investigated. For this purpose analyses of the soluble and insoluble nitrogenous constituents were made, so that it is possible to give some account of the changes of amino-acids and proteins. It has been found consistently in experiments with both yeast and root tissues that glutamic acid and its amide, glutamine, are rapidly formed in the early stages of assimilation, corresponding fairly closely in time with the highest rates of cell oxidation. The results of an experiment in which yeast cultures were supplied with ammonium phosphate are given in Figure 3.

During the first 30 min. a marked increase of glutamic acid and glutamine occurs with a smaller accumulation of alanine; together these constituents account for about 70 per cent. of the total nitrogen assimilated by the cells over the initial period. Subsequently, they are maintained at a fairly steady or falling level. There is a gradual formation of other, as yet unidentified, soluble–N, and a small increase in the tripeptide, glutathione, was observed in some of these experiments (Yemm and Folkes, 1954). The progressive rise in complex insoluble–N in the cells indicates an active synthesis of protein during the course of the experiment.

At present, the identification of amino-acids and amides in the yeast rests mainly on separations by paper chromatography, or on the use of specific enzymes for analysis. Most of the estimates of glutamic acid and glutamine were made by means of glutaminase and glutamic decarboxylase, prepared from *Clostridium welchii* by the method described by Krebs (1948).

Analytical data from a similar experiment with barley roots are shown in Figure 4. On a much longer time-scale they have several features in common with the data for yeast. Ammonia–N accumulates temporarily in the roots, but at first the main product of assimilation is glutamine, which makes up about 80 per cent. of the ammonia utilized in the first 12 hours. Asparagine, the other common plant amide, increases at a later stage; together the two amides account for almost all of the free amino–N in the tissues.

In some of the experiments with barley roots it has been possible to obtain more decisive evidence of the primary synthesis of amides from ammonia by using isotopic nitrogen to trace the products of assimilation in the cells. Ammonium phosphate, containing about 30 per cent. excess of ^{15}N, was supplied to the roots and its incorporation into the amide and other nitrogen fractions was estimated after varying periods of assimilation. The abundance of the isotope in some of the different fractions is shown in Figure 5.

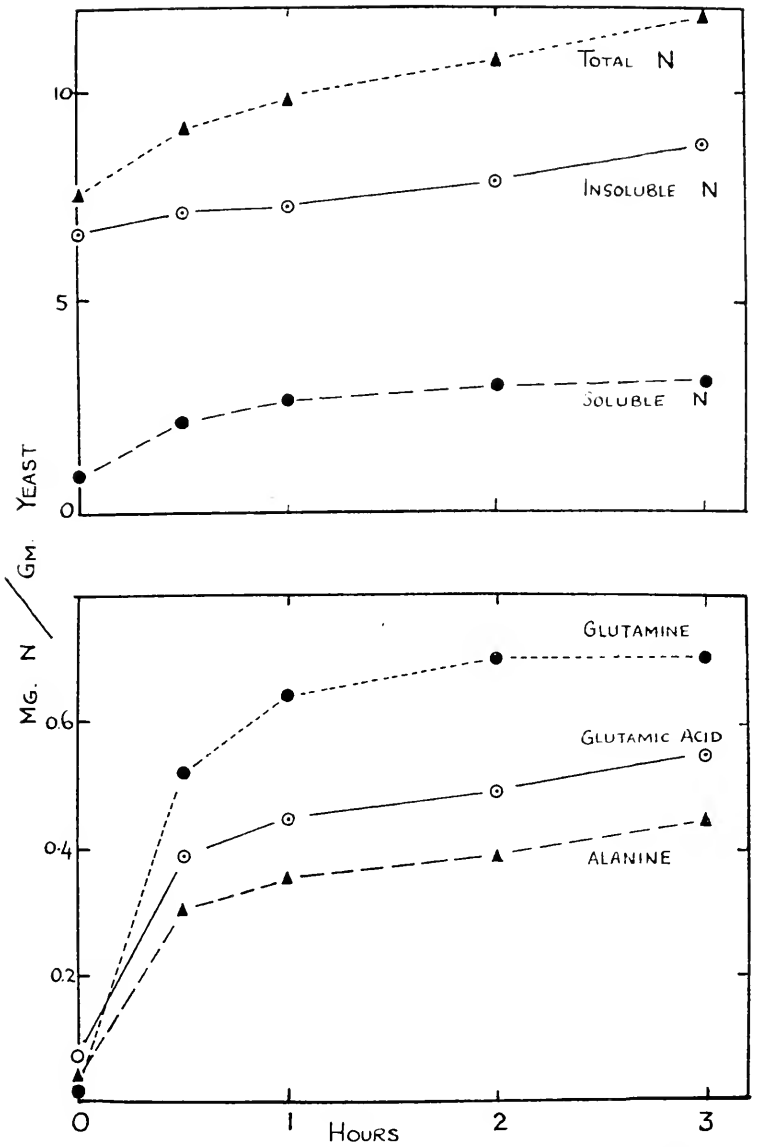


Figure 3. Changes in nitrogenous constituents during the assimilation of ammonia –N by yeast. Total nitrogen, insoluble (protein) and soluble fractions are shown in the upper part of the figure, and the chief amino-acids and amides in the lower part.

It is clear that ^{15}N supplied as ammonia is quickly incorporated into glutamine; the abundance in the amide approaches that of the ammonia–N in the tissues after $10\frac{1}{2}$ hours, thus providing direct evidence of a primary synthesis. Asparagine amide, on the other hand, has a lower abundance which gradually rises during assimilation; it is possible that this amide is formed secondarily from glutamine. The protein–N

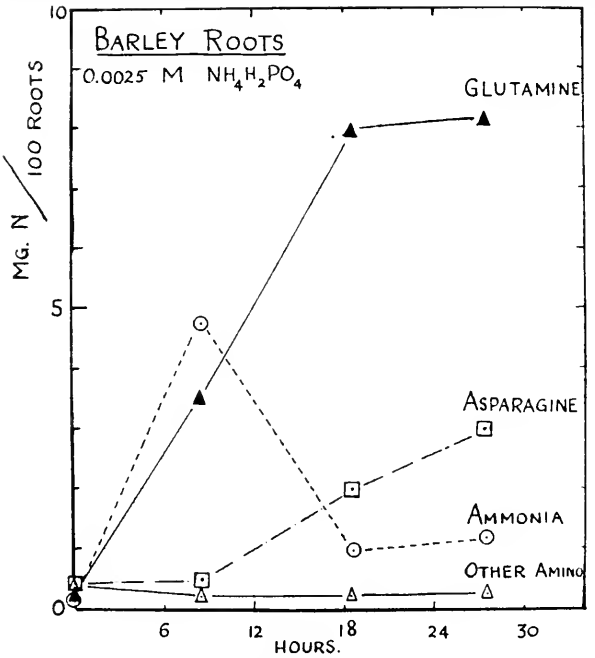


Figure 4. Changes of amino-acids and amides in excised barley roots during the assimilation of ammonia–N. Samples of 40 root systems were analysed after varying periods of assimilation in aerated culture solutions containing ammonium phosphate.

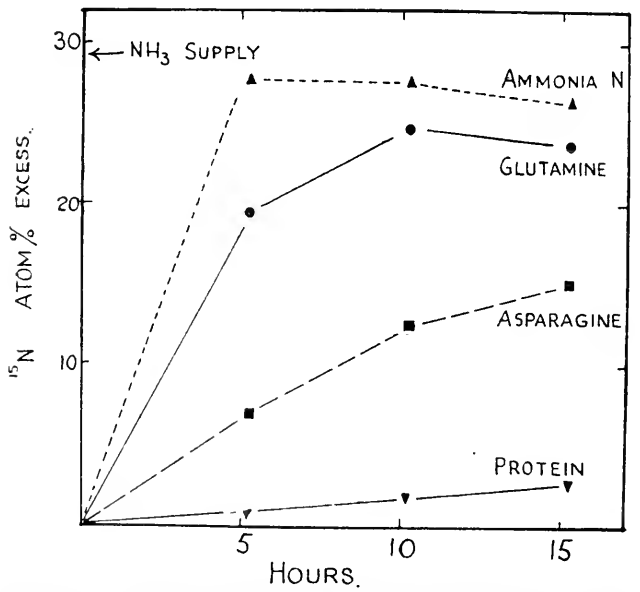


Figure 5. The incorporation of isotopic nitrogen into the nitrogenous constituents of excised barley roots. Ammonium phosphate containing 29·3 atom per cent. excess ^{15}N was supplied to the roots, and the separated fractions subjected to analysis in a mass spectrometer.

of the tissues shows a fairly steady rate of incorporation of ^{15}N, which is much greater than can be accounted for by the net synthesis of protein in the roots. It seems probable, therefore, that the proteins of the cells are maintained in dynamic equilibrium with soluble nitrogenous constituents by means of exchange or other reactions.

Much other work, reviewed by Chibnall (1939), by Steward and Street (1947) and by Virtanen and Rautenen (1952) converges with that discussed above in showing that the amides, asparagine and glutamine, may be readily formed from ammonia in plant cells. The more active role of glutamine in protein metabolism is indicated by earlier experiments of Yemm (1937, 1949, 1950), Steward and Street (1946), Rautenen (1948) with higher plants, and by those of Roine (1947) and Virtanen, Csarky and Rautenen (1949) with yeast. Vickery, Pucher, Schoenheimer and Rittenberg (1940) and MacVicar and Burris (1948), using isotopic nitrogen, have shown that glutamic acid and glutamine are highly active in the metabolism of proteins in plants.

THE BREAKDOWN OF CARBOHYDRATES IN RELATION TO RESPIRATION AND THE SYNTHESIS OF AMINO-ACIDS

As already indicated, a rapid depletion of carbohydrate accompanies the high rate of respiration during the assimilation of nitrogen by the cells. In most of the experiments, analytical data were obtained from which it is possible to estimate the losses of readily available carbohydrates. As no external supplies of carbohydrate were provided, these losses can be related to respiration and the synthesis of nitrogenous constituents. For this purpose balance sheets for carbon have been drawn up, in which the production of respiratory CO_2 and the synthesis of amino-acids are balanced against the breakdown of carbohydrates. An example of the data from a typical experiment with barley roots is given in Table I. It is evident that the breakdown of carbohydrates, mainly hexoses and sucrose in these tissues, is adequate to

TABLE I

Carbon balance sheet for barley roots

Roots excised from 10-day-old seedlings and allowed to assimilate for 18 hours in 0·0025 M $NH_4H_2PO_4$ at 22·5° C.

Products	mg. c/100 roots
(1) Respiratory CO_2	36·6
(2) Synthesis of Glutamine	15·9
(3) Synthesis of Asparagine	2·8
Total (1), (2), (3)	55·3
Loss	
Carbohydrates	61·7

meet the needs for both the synthesis of amides and the production of CO_2. The losses of carbohydrates during the eighteen hours of assimilation are in fact slightly greater

than the total requirement and there is every indication that the carbon skeletons for the syntheses of the amino-acids and amides are provided in this way.

Data obtained from similar experiments with yeast are summarized in Table II. In the yeast a much more active synthesis of amino-acids and proteins occurs, but here again there is evidence that this, together with the respiratory losses, is mainly met by the breakdown of the reserve carbohydrates, glycogen and mannans. Other sources of carbon in the cell are drawn upon to a less extent; measurements of the respiratory quotient suggest that this may be from fat reserves. There are several other points of interest in these records: they show, for example, the very great drain on the reserves associated with nitrogen assimilation so that the diversion of carbon

TABLE II

Carbon balance sheet for yeast

Aerated cultures allowed to assimilate for 2 hours in 0·01 M $NH_4H_2PO_4$ at 25° C.

Products	mg. C/1 gm. Yeast
(1) Respiratory CO_2	5·9
(2) Syntheses	
Glutamine	2·2
Glutamic acid	1·1
Alanine	0·5
Other sol. N	1·9
Protein	6·6
Total (1) and (2)	18·2
Losses	
Carbohydrates	13·8
Other (by difference)	4·4

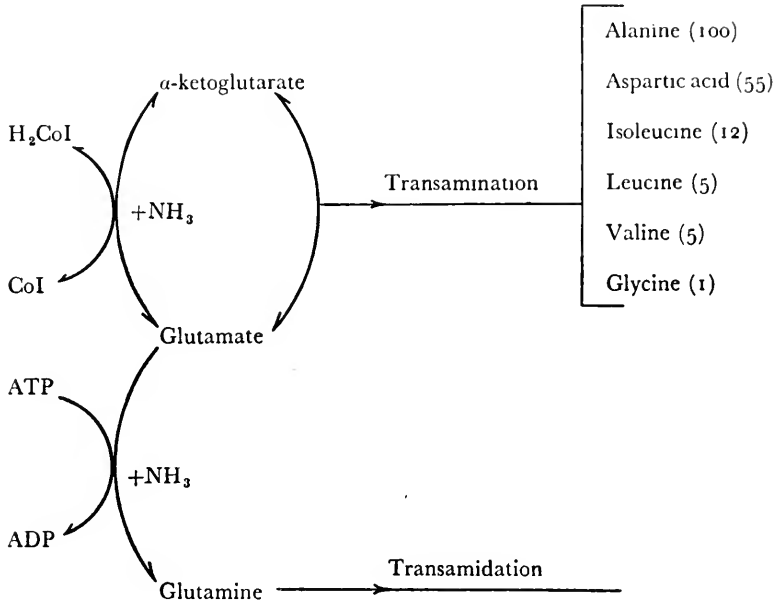
to the synthesis of amino-acids, amides and proteins is several times greater than that lost as carbon dioxide.

To sum up, the chief results of these analytical experiments indicate that the formation of glutamic acid and its amide, glutamine, occurs in the first phases of nitrogen assimilation in both yeast and root tissues. The synthesis is associated with high rates of cell oxidation and is sustained by the mobilization of carbohydrate and possibly other reserves in the cells.

THE METABOLISM OF GLUTAMINE

A close coupling between the formation of glutamine and the metabolism of carbohydrates may be inferred from the enzymic mechanisms associated with the synthesis of the amide. It is highly probable that glutamic acid arises in the cell by reductive

amination of α-ketoglutaric acid, followed by further combination with ammonia to give the γ-amide. The course of the synthesis is outlined below.



Some evidence of the occurrence of these reactions in barley seedlings has been gained by the separation of enzymes from the young embryos. Highly active preparations of glutamic acid dehydrogenase, which catalyses the reductive amination of α-ketoglutarate linked with the oxidation of pyridine nucleotide (CoI), have been obtained from the seedlings. With yeasts similar preparations of the dehydrogenase, but reacting with coenzyme II, have been obtained by Adler and others (1938), while Elliott (1951) has demonstrated the enzymic synthesis of glutamine coupled with a conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP).

The dependence of amide synthesis on phosphorylation has been further indicated in the present experiments by the action of 2.4–dinitrophenol. This cell poison strongly inhibits the synthesis of glutamine at low concentrations ($0{\cdot}6–2{\cdot}5 \times 10^{-5}$ M at pH 5·5), although at these levels the rate of oxygen uptake is unaffected, or slightly increased. This typical uncoupling action is attributable to the selective action of dinitrophenol on the phosphorylations linked with cellular oxidations (Simon, 1953).

The enzymic systems engaged in the synthesis of glutamine may readily account for its close co-ordination with carbohydrate metabolism and respiration. α-Ketoglutaric acid, the organic acid precursor, is an intermediary in the oxidation of carbohydrate by the tricarboxylic acid cycle, while pyridine nucleotides and adenosine triphosphate occupy key positions as electron and phosphate carriers respectively in cell oxidations. These direct links with the exergonic reactions of respiration may form the starting-point in the synthesis of other amino-acids and of peptides by transfer reactions, such as transamination and transamidation, which proceed with relatively little change

of free energy. Steward and Street (1946, 1947), Yemm (1949), Fruton (1950), Hanes *et al.* (1950), and Waelsch (1952) have discussed the potentialities of glutamine in the canalizing of energy to protein synthesis.

Transaminases, which promote the transfer of α-amino groups from glutamic acid to other α-keto acids, are known to be widely distributed in higher plants (Leonard and Burris, 1947) and their presence in food yeast was demonstrated by Roine (1947). A preliminary investigation of these enzymes in young barley seedlings has shown that they provide a mechanism for formation of at least six other amino-acids, as indicated above. Estimates of the relative rates of transamination with the different amino-acids are given in the diagram. It is of interest that the glutamic–alanine and glutamic–aspartic systems give the highest activities, which may account for the formation of alanine and asparagine during the rapid assimilation of nitrogen.

The nature of the transamidation reactions and their significance in the biosynthesis of peptides and proteins is at present uncertain. However, Dowmont and Fruton (1952) have found that plant proteinases, such as papain and ficin, catalyse the synthesis of peptide bonds from amides by transamidation, so that, in artificial systems, formation of polypeptide structures occurred. Participation of the γ-amide group of glutamine in transfer reactions in the cell is indicated by the occurrence of glutamyl transferase in micro-organisms (Grossowicz, Wainfan, Borek and Waelsch, 1950) and in higher plants, (Stumpf, Loomis and Michelson, 1951). In this connexion preparations of glutamyl transferase have recently been made from barley seedlings and the activity estimated in model systems by measuring the rate of replacement of the amide group of glutamine by hydroxylamine. The activity of the enzyme in cell-free preparations indicates that it could play a substantial part in peptide synthesis: the rate of transfer of amide groups observed in cell-free preparations is, in fact, adequate to account for the high rates of peptide synthesis which occur in the young embryo.

The products of the action of γ-glutamyl transferase in the cell are not yet known. The work of Hanes and others (1950, 1952) has suggested that the tripeptide, glutathione, which is very widely distributed in living cells, may take part in transpeptidations involving the transfer of γ-glutamyl groups. But, under the conditions so far tested, the tripeptide is inactive with the glutamyl transferase of barley and, in yeast, the changes of glutathione during assimilation of nitrogen are relatively small, as already indicated. On the other hand, there is some evidence that the formation of glutathione may be correlated with protein synthesis in the early stages of the development of barley embryos. Estimated by means of the nitroprusside reaction of Grunert and Phillips (1951), the peptide increases markedly at a time when synthesis of protein is beginning, as shown by the results given in Figure 6.

Mainly in the reduced form, glutathione accumulates in the tissues after about two days' germination and at the same time there is an acceleration of protein synthesis. Histochemical tests indicate that it occurs mainly in the meristematic regions, which are in all probability very active in the synthesis. However, it is possible that the action of the tripeptide in oxidation-reduction systems of the cell, recently elucidated by the work of Conn and Vennesland (1951) and Mapson and Goddard (1951), may account for this relation. Moreover, glutathione represents only a very small part of the total soluble nitrogen of the embryo, and other analyses suggest the

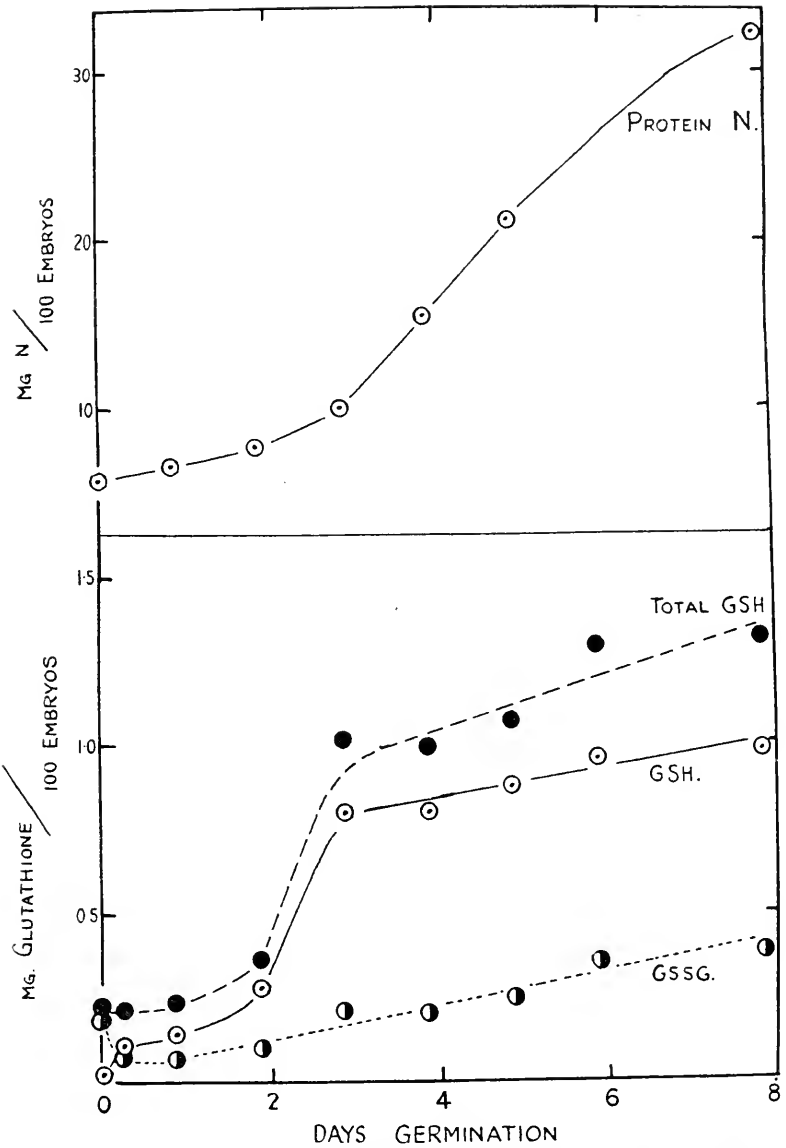


Figure 6. Changes of glutathione during the development of barley embryos. Samples of 50–100 embryos were extracted with 2·5 per cent. sulphosalicylic acid; glutathione (GSH) was estimated by the nitroprusside reaction, before and after reduction on a mercury cathode. Changes of total insoluble N (protein) are shown in the upper part of the figure.

presence of appreciable quantities of other peptides, which have not as yet been characterized. The study of these peptides, and particularly of their rate of turnover during the assimilation of nitrogen, may provide more decisive evidence concerning the mechanisms of protein synthesis in the cells.

CONCLUSION

With regard to the wider problems of protein synthesis, the following conclusions may be drawn from the data so far obtained.

(1) A rapid formation of glutamic acid and glutamine, which occurs in the first stages of nitrogen assimilation in yeast and in barley tissues, is closely coupled with carbohydrate metabolism and the exergonic reactions of cellular oxidations.

(2) The primary synthesis of amino and amide groups may be linked with the formation of other amino-acids and of peptides by means of enzymic systems which promote transamination and transamidation in the cell.

(3) Some support is therefore given to the hypothesis of peptide-bond formation by transamidation and transpeptidation, but as yet very little is known of the specificity or course of peptide synthesis in living cells.

(4) It seems possible from this and other evidence that the action of specific surfaces, visualized in the 'template' hypothesis, operates at a later phase of protein synthesis by affecting the folding and cross-bonding of polypeptide structures. The role of nucleic acids in protein formation may be in this stage, rather than in the direct synthesis of peptide bonds.

The experimental work was carried out in collaboration with my colleagues, Dr. Folkes and Dr. Willis; their permission to present some hitherto unpublished results is gratefully acknowledged.

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Discussion

Chairman: J. Brachet

G. Pontecorvo. Your results on transamination of glutamic acid to form other amino-acids in the heirarchic order shown are exactly the same as those found by Fincham and others by the less orthodox but more efficient method of using mutants in micro-organisms. This supports your conclusion as to the general occurrence of such processes.

E. W. Yemm. The investigation of transamination in barley embryos is not yet complete. The relative activities given are based on comparative measurements in cell-free preparations without addition of pyridoxal phosphate. From the work of Cohen it seems possible that other transaminations may be detectable after reinforcement of the preparations by addition of the coenzyme.

W. S. Reith. It is very interesting to see this striking difference in the relative amounts of glutamine and asparagine. We have found in the growing cells of bean roots just the opposite situation. There the amount of asparagine greatly exceeds that of glutamine. We interpreted this as an accumulation of asparagine while the glutamine was rapidly depleted owing to its active participation in transaminations.

As for protein-nitrogen determinations, I should like to point out that we find that very misleading results can be obtained from trichloracetic acid precipitates. Such protein precipitates can contain a great amount of non-protein nitrogen.

E. W. Yemm. The relation between the two amides, glutamine and asparagine, in the metabolism of barley plants has been discussed in an earlier account of our work. In general asparagine accumulates in the cell under conditions of carbohydrate shortage and proteolysis; this seems to hold for root tissues. Under conditions normally obtaining during the growth of the plant glutamine appears to be much more closely related to the metabolism of proteins.

The estimates of total insoluble–N (protein) in roots and yeast were usually obtained after extraction with alcohol and water.

O. Maaloe. Is it possible, in your system, to follow synthesis of amino-acids, peptides, and protein long enough to observe an equilibrium between the concentrations of low- and high-molecular-weight compounds; if so, can it be estimated what fraction of amino–N, at equilibrium, is in the pool of low-molecular-weight substrates for protein synthesis?

E. W. Yemm. Equilibrium conditions between the nitrogenous constituents do not appear to be established in our experiments; but we have very little knowledge of the nature or amount of peptides present in the cells.

W. S. Reith. In the meristematic cell, the amount of peptide nitrogen and amino-acid nitrogen is very small in comparison with the protein nitrogen.

B. F. Folkes. The low level of soluble nitrogen other than glutamic acid, glutamine or alanine, indicates the low level of other amino-acids and peptides in the cell. It seems that the low level of these products limits the rate of protein synthesis.

L. Rinaldini. The rise in GSH might be connected with the oxygen uptake in view o the respiratory mechanism recently found in plants by Mapson, where GSH acts as a hydrogen carrier between dehydrogenases and ascorbic acid, which in turn reacts with molecular oxygen.

E. W. Yemm. I fully agree that glutathione may be active in other processes of cellular metabolism. In addition to transpeptidations and oxidation-reductions it may have a regulating action on –SH enzyme systems.

E. Ambrose. With regard to the transpeptidation and template theories of protein synthesis, if the transpeptidation theory is correct, there is a pool of peptides in dynamic equilibrium within the cells, which is increased in concentration by feeding with the source of nitrogen; this increase may be to some extent independent of other cellular processes. If on the other hand we are dealing with a nucleic acid template, there might be a close correspondence between the concentration of peptides and of nucleic acids within the cell. Has a relationship been found between the peptides and nucleic acid concentrations within yeast cells?

E. W. Yemm. We have not yet studied the change of nucleic acids during protein synthesis in food yeast; as far as I am aware no data have been published. Judging from Gale's work with bacteria and Hokin's with animal tissues, substantial synthesis of proteins may occur in cells without appreciate changes in the amount of nucleic acids.

J. F. Danielli. The fact that more isotopic nitrogen appears in the proteins than can be accounted for by net synthesis may mean that individual amino-acids or peptides

are exchanging with the protein. Is there any evidence that this is so, and if so which amino-acids are concerned?

E. W. Yemm. The distribution of ^{15}N in the proteins of barley roots has not been examined in detail. However, with leaf-tissue proteins we have evidence that the isotope is incorporated to the greatest extent in glutamic, aspartic and amide nitrogen of the protein, although there are appreciable amounts in the monocarboxylic and basic amino-acids. From this and other work it seems probable that the abundance of ^{15}N in the different amino-acids of the tissue proteins reflects the extent to which the amino-acid becomes labelled in the metabolic pool. In both plant and animal tissues, supplied with isotopic ammonia, incorporation is usually greatest in glutamic, aspartic and amide nitrogen, probably owing to the ease with which these are synthesized from ammonia.

A. J. Willis. The incorporation of ^{15}N into the protein of barley roots is much more extensive in the amide groups than in the total nitrogen of the protein. This indicates extensive exchange reactions involving these amide groups.

J. Brachet. In connexion with Dr. Yemm's suggestion that there might be two different mechanisms involved in protein synthesis (transpeptidation and template activity), it is worth pointing out that Koritz and Chantrenne recently obtained evidence for such a viewpoint: in reticulocytes, incorporation of labelled amino-acids precedes the peak in RNA synthesis; this peak coincides with the formation of various enzymes, which might be produced by a specific template mechanism.

The biosynthesis of pentoses and their incorporation into mononucleotides

by

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THE importance of mononucleotides both as building-blocks of the nucleic acids and as constituents of a number of coenzymes for reactions in intermediary metabolism is generally accepted. An understanding of the mechanism by which the mononucleotides are formed might therefore be of significance for the explanation of various biological phenomena. I should like to discuss possible pathways by which mononucleotides may be formed, and also to mention the present evidence for the pathways of the biosynthesis of the sugar part of the nucleotides, i.e. the ribose.

In the last few years considerable knowledge has accumulated about enzyme reactions leading to ribose phosphate formation. These new facts have been obtained mainly from experiments on the oxidative breakdown of carbohydrates. By this term we are accustomed to mean the extremely important oxidative cycle of Krebs. The existence of an alternative pathway of carbohydrate oxidation was, however, indicated by work of Warburg and Christian (1937), Lipmann (1936) and Dickens (1936).

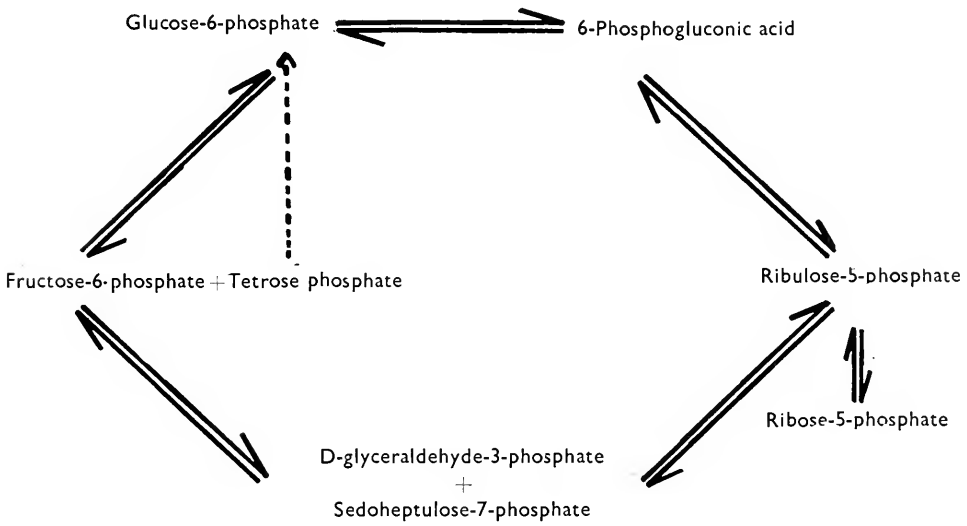

Figure 1. *The oxidative cycle.*

Recent studies of this alternative pathway have revealed the existence of a new cycle for the oxidative breakdown of carbohydrates. This cyclic mechanism has been established primarily by Horecker and his group, and has been formulated in the following way (Horecker, 1953).

In this reaction scheme the oxidation of glucose-6-phosphate to the δ-lactone of 6-phosphogluconate is catalysed by Warburg's well-known *Zwischenferment*. The further breakdown of 6-phosphogluconate has been found to be an oxidative decarboxylation leading to the formation of the five-carbon keto sugar ribulose-5-phosphate. Both of these oxidation steps require triphosphopyridine nucleotide as hydrogen acceptors. To account for the formation of ribulose-5-phosphate it has been postulated that 6-phosphogluconate is first oxidized in the 3-position. A free 3-keto phosphogluconate has, however, not been isolated as an intermediate, and the possibility exists that both oxidation and decarboxylation are catalysed by the same enzyme as is the case with some other oxidative decarboxylations. The ribulose-5-phosphate can be converted by a pentose phosphate isomerase to ribose-5-phosphate, a reaction which is completely analogous to the interconversion of fructose-6-phosphate and glucose-6-phosphate. These two pentose phosphate esters can now interact, and with a highly purified enzyme the product has been shown in addition to glyceraldehyde-3-phosphate to be a phosphate ester of the seven-carbon keto sugar, sedoheptulose. This sugar was first isolated from the sedum plant, where it is present in large amounts (La Forge and Hudson, 1917). Recently Calvin and his group (Benson *et al.*, 1951) have found that sedoheptulose phosphate is one of the earliest products to be formed during photosynthesis, a fact which is a further indication of its importance in the intermediary metabolism. Glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate can now further interact, and in the presence of the enzyme transaldolase the products are fructose-6-phosphate and a tetrose phosphate. This reaction has been proved to be a transfer of the three first carbons of sedoheptulose-7-phosphate, i.e. the dihydroxyacetone group, to glyceraldehyde-3-phosphate, whereby fructose-6-phosphate is formed by an aldole condensation. Fructose-6-phosphate is then converted by hexose phosphate isomerase to glucose-6-phosphate, and we are back at the starting-point of the cycle. Thus, with two turns of the cycle two moles of CO_2 are evolved, and four moles of triphosphopyridine nucleotide are reduced, which will require two moles of O_2 for oxidation. At the same time one mole of glucose-6-phosphate is regenerated, and one mole of tetrose phosphate is formed. This tetrose may, moreover, be further converted to hexose monophosphate by a mechanism not yet completely clarified, whereby the cycle is completed (Horecker, 1953, Horecker *et al.*, 1954). It should furthermore be emphasized that all of the reactions of the cycle have been shown to be reversible. The activity of some of the enzymes involved in this scheme has been investigated in a variety of normal mammalian tissues and in tumours (Glock and McLean, 1954), and the quantitative significance of the oxidative pathway has been investigated with isotopically labelled compounds in several organs (Bloom, Stetten and Stetten, 1953).

This system of enzyme reactions then furnishes us with two processes for pentose formation, i.e. the direct oxidation of glucose-6-phosphate to ribulose-5-phosphate and ribose-5-phosphate, and the reaction between one molecule of glyceraldehyde-3-phosphate and one molecule of sedoheptulose-7-phosphate leading to the formation

of two molecules of pentose phosphate. This latter reaction has been studied with highly purified enzymes from liver, spinach (Horecker *et al.*, 1953) and yeast (Racker *et al.*, 1953). Several possibilities obviously exist for mechanisms by which sedoheptulose phosphate may be formed from pentose phosphates, but conclusive evidence indicates (de la Haba *et al.*, 1953; Horecker and Smyrniotis, 1953; Racker *et al.*, 1953) that it is formed by a condensation between a two-carbon compound and a five-carbon compound, and that it is the ribulose-5-phosphate which is donator of the two-carbon compound. The latter, which would be at the oxidation level of glycol aldehyde, then combines with ribose-5-phosphate to form the sedoheptulose-7-phosphate. Free glycolaldehyde, however, is not active nor does it accumulate in any of these reactions. The sedoheptulose phosphate formation has, therefore, been visualized as an acetoin condensation between an activated form of glycolaldehyde and ribose-5-phosphate. This is consistent with the thiamine pyrophosphate requirement of the reaction which has been formulated as follows:

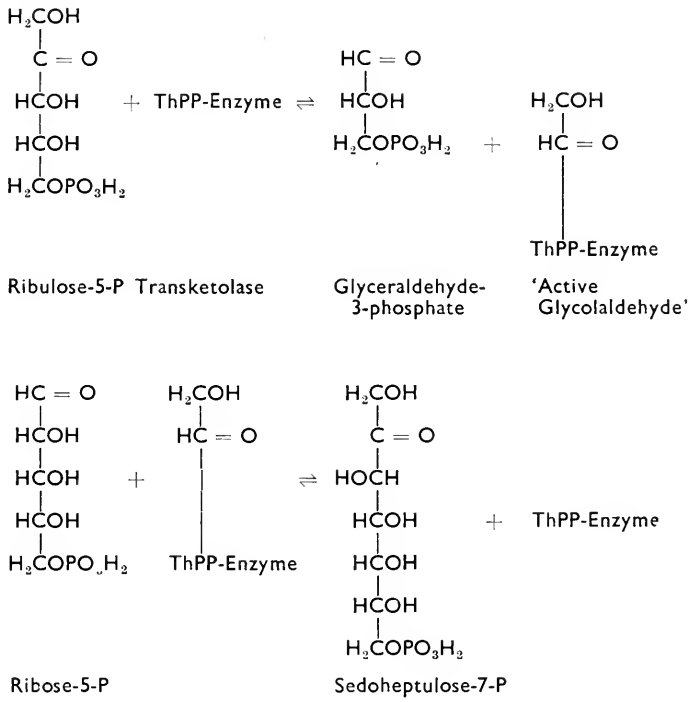

Figure 2. The formation of sedoheptulose-7-phosphate in the transketolase reaction. (From Horecker et al., *1953.)*

Since the enzyme catalyses the transfer of ketol linkages, it has been named transketolase. Thus, in these reactions the keto sugar esters, ribulose-5-phosphate and sedoheptulose-7-phosphate, serve as donors of 'active glycolaldehyde', and the aldo sugar esters, ribose-5-phosphate and glyceraldehyde-3-phosphate, serve as acceptors of 'active glycolaldehyde'. Also several other compounds can serve as substrates in these reactions. Those known at the present time are listed in Table I,

and still others may be found. The transketolase reaction then provides us with a system by which pentoses can be formed by condensation between a two-carbon and a three-carbon fragment. A third mechanism for pentose formation is suggested by work of Hough and Jones (1953) who found xylulose phosphate to be formed from triose phosphate and dihydroxymaleic acid in the presence of an enzyme from peas. The details of this mechanism seem, however, not to be entirely clear yet.

We have now accounted for some enzyme reactions for pentose formation. But how are the pentoses actually formed in the intact organism? By which mechanism are the pentoses in the nucleic acids formed? The best tool for getting such information is obviously ingestion of isotopically labelled compounds, the fate of which can be followed. In the case of ribose the pattern of labelling of the carbon atoms of the pentose of the nucleic acids obtained in this way may give valuable information.

TABLE I

'Active glycolaldehyde' donors and acceptors

'Active glycolaldehyde' donors		'Active glycolaldehyde' acceptors	
Ribulose-5-phosphate	*a,b*	*D*-Glyceraldehyde-3-phosphate	*a,b*
Sedoheptulose-7-phosphate	*a*	Ribose-5-phosphate	*a,b*
L-Erythrulose	*a*	Glycolaldehyde	*b*
Hydroxypyruvic acid	*a,b*		
		L-Glycolaldehyde-3-phosphate	*a*
		D-Glyceraldehyde	*a*

a Horecker *et al.* (1953). *b* Racker *et al.* (1953).

Such experiments have been performed by Bernstein (1953). The concept which led to these experiments was the following: If the pentoses were formed by the oxidative breakdown of glucose-6-phosphate by removal of number one carbon or possibly by removal of number six carbon by decarboxylation of a hexuronic acid, the distribution of the tracer in the ribose should be similar to that of the remaining five carbons of the hexose. A deviation from this picture would indicate the involvement of some other synthetic mechanism. The liver glycogen and the ribose from the nucleic acids of the internal organs of chicks were therefore isolated after feeding with different ^{14}C-labelled compounds. The glycogen and the ribose were degraded biologically and chemically, and the specific activity of each carbon was determined. Assuming that the ^{14}C pattern of glycogen corresponds to that of glucose-6-phosphate during the experiment, and that the ^{14}C pattern of the ribose is not altered when the nucleic acids are formed, Bernstein compared the relative pattern of ^{14}C labelling in the glucose and in the ribose. As can be seen from Table II the pattern of labelling of the ribose does not in any case correspond with that of the 5 carbons in succession of the glucose derived from glycogen. However, pentose formation by a condensation of a two-carbon with a three-carbon compound is consistent with the results obtained. $C_{(3)}$, $C_{(4)}$ and $C_{(5)}$ of the pentose should then arise from the same triose which is the precursor of glycogen. The $C_{(1)}$ and $C_{(2)}$ of the pentose could be derived from $C_{(1)}$

and $C_{(2)}$ of glycogen. These findings, therefore, are by no means in disagreement with the reaction catalysed by the transketolase. Similar experiments performed with *E. coli* suggest that the oxidative decarboxylation of 6-phosphogluconic acid is the primary pathway for pentose formation in this organism (Cohen, 1951; Sowden *et al.*, 1954).

We have now seen by which possible mechanism ribose may be formed in living organisms. But by which reactions are the ribose phosphates linked to the purines and pyrimidines to form the nucleotides, the building blocks of the nucleic acids? About eight years ago Kalckar (1947) demonstrated the enzymatic synthesis o

TABLE II

Relative ^{14}C *distribution in ribose and glycogen of chicks fed* $^{14}CH_2NH_2COOH$

Experiment no.	Compound analysed	Carrier dilution	Relative specific activity					
			$C_{(1)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(5)}$	$C_{(6)}$
3	Glycogen	7·4	100	133	16	16	133	100 (9940)
	Ribose	17·0	79	98	27	124	100 (222)	

The figures in parentheses indicate the level of specific activity (counts per minute per millimole of carbon) at which the determination was actually made for the carbon assigned a value of 100 (From I. A. Bernstein (1953) *J. Biol. Chem.* **205**, 317).

nucleosides with the nucleoside phosphorylase system. The reaction is the well-known reversible phosphorolytic cleavage of nucleosides to free base and ribose-1-phosphate. The equilibrium of the reaction:

Hypoxanthine riboside + orthophosphate ⇋ hypoxanthine + ribose-1-phosphate

is in favour of the synthesis of the riboside. High enzyme activity is present in both mammalian organisms and in micro-organisms. The enzyme is active towards a number of different purine nucleosides and also towards purine deoxynucleosides (Friedkin, 1953). Nicotinamide riboside is attacked by an enzyme which is probably identical with the enzyme of Kalckar (Rowen and Kornberg, 1951). Pyrimidine nucleosides, however, can be split by an apparently different phosphorylase (Lampen, 1952). Thus, with these reactions we can account for the formation of the linkage between ribose and several of the nitrogen bases of the nucleic acids. The nucleosides formed in this way might then be phosphorylated to nucleotides by a kinase reaction, i.e. with ATP as phosphate donor. Such reactions have been demonstrated by Kornberg and Pricer (1951). They found that both adenine riboside and 2-amino adenine riboside can be phosphorylated to the corresponding 5′-nucleotides. The enzyme is, however, strictly specific with regard to adenosine and 2-amino adenosine, and other nucleoside kinases have not been found yet.

A more unspecific reaction for nucleotide formation was found by Brawerman and Chargaff (1953). They showed that some unspecific phosphatases have transferase activity also with nucleosides as acceptors. With phenyl phosphate as phosphate donor they found that all possible nucleotides could be formed from the corresponding nucleosides in the presence of prostate phosphatase, while a phosphatase from malt under some conditions catalysed the formation of only 5′-nucleotides. This type of transfer reaction, however, might not play a quantitatively significant role under physiological conditions, at least when growth is involved, as it often requires a high substrate concentration and still gives a fairly low yield.

An entirely different pathway for nucleotide formation was suggested by some experiments performed with ^{14}C-labelled formate (Greenberg, 1951). Formic acid is known to be incorporated into the purine ring, and Greenberg was able to isolate labelled hypoxanthine, inosine and inosinic acid from pigeon-liver extracts, which had been incubated with labelled formic acid. But the interesting part of this observation was that the specific activity of inosinic acid was significantly higher than that of inosine and hypoxanthine. In other words, inosine-5′-phosphate was probably the primary product, and both the nucleoside and the free base were probably degradation products of the nucleotide. Similarly Leder and Handler (1951), working with nicotinamide nucleotide synthesis in erythrocytes, found evidence for bypassing of the nucleoside stage. Consistent with this concept Buchanan and his group (Williams and Buchanan, 1953) also found evidence for bypassing of inosine in the formation of inosinic acid from hypoxanthine. They furthermore found that synthesis of inosinic acid was considerably activated by addition of ribose-5-phosphate and adenosine triphosphate to pigeon-liver extract. The reaction was shown to be catalysed by at

TABLE III

Reactivation of a dialysed extract of pigeon liver

Additions	Counts/min. in the acid-soluble nucleotides
Undialysed extract, 0·5 ml.	2,150
Dialysed extract, 0·5 ml.	15
+ 3 μM ribose-5-phosphate + 1 μM ATP	1,860
+ 3 μM ribose-5-phosphate	82
+ 1 μM ATP	22
+ 3 μM ribose-1-phosphate + 1 μM ATP	2,250
+ 3 μM ribose-′3′-phosphate + 1 μM ATP	53
+ 5 μM deoxyribose-1-phosphate + 1 μM ATP	30
+ 5 μM ribose + 1 μM ATP	90
+ 1 μM AMP	15
+ 1 μM inosinic acid	15

7γ — 7,000 counts/min. of 8-carbon-14-adenine added in every case.
(From Saffran, M. and Scarano, E. (1953). *Nature, Lond.* **172**, 949.)

least two enzymes which could be separated by alcohol fractionation. The first enzyme reaction consisted in a reaction between ribose-5-phosphate and adenosine triphosphate to yield an activated ribose phosphate ester.

In Dr. Kalckar's laboratory similar observations have been made. Adenine is known to be incorporated into the nucleic acids on a large scale (Brown, 1948), and Goldwasser (1953) found that in pigeon-liver extract ^{14}C-labelled adenine is incorporated into adenosine monophosphate, adenosine diphosphates and adenosine triphosphates at an appreciable rate. Saffran and Scarano (1953) working with the

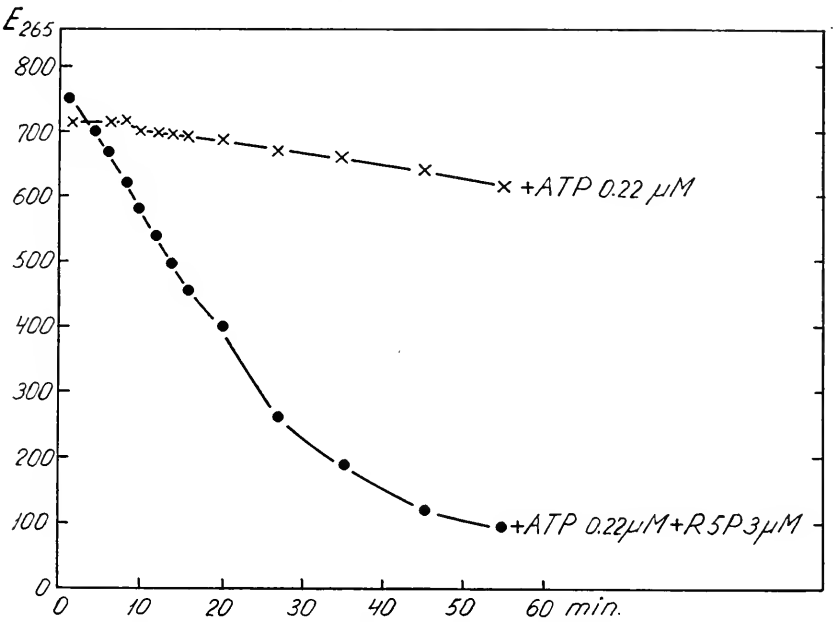

Figure 3. *Phosphorylation of ribose-5-phosphate with adenosine-triphosphate.*
The reference cuvette contained 0·22 μM adenosine triphosphate in 3 ml., and the spectrophotometer was brought to zero at an optical density of 0·5. The experimental cuvettes contained 0·39 mM potassium chloride, 0·03 mM magnesium chloride, 0·045 mM dipotassium hydrogen phosphate, 5-adenylate kinase 20γ of protein per ml., 5-adenylate deaminase 25γ of protein per ml., total volume 3·19 ml., pH 6·8. At 0 min. 11γ of protein per ml. of 5-phosphoribokinase was added to the experimental cuvettes
Abscissa: time in minutes; ordinate: extinction × 10^3 at 265 mμ. From Scarano (1953).

same system found that here also the presence of ribose-5-phosphate and adenosine triphosphate stimulated the incorporation of adenine into adenosine monophosphate. They found furthermore that in the dialysed extract the incorporation was completely dependent on both of these two compounds (Table III). In this system ribose-5-phosphate could, however, be replaced by ribose-1-phosphate, whereas ribose-2-phosphate and ribose-3-phosphate were inactive. In addition they were able to demonstrate that this reaction also proceeds in at least two steps, the first one being an activation of ribose phosphate with adenosine triphosphate, and the second one being the reaction between this activated compound and adenine to form the 5′-adenylic acid.

The first enzyme which activates ribose-5-phosphate was found to be fairly heat stable and was partly fractionated. This enzyme fraction was shown among a number of sugars and sugar phosphate esters to utilize adenosine triphosphate in the presence only of ribose-5-phosphate or fructose-6-phosphate (see Figure 3) (Scarano, 1953).

All these experiments suggest that the nucleosides may to a great extent be by-passed in the synthesis of the nucleotides, and that a special type of ribose phosphate ester is an intermediate in this synthesis. Here it was natural to consider a ribose-1,

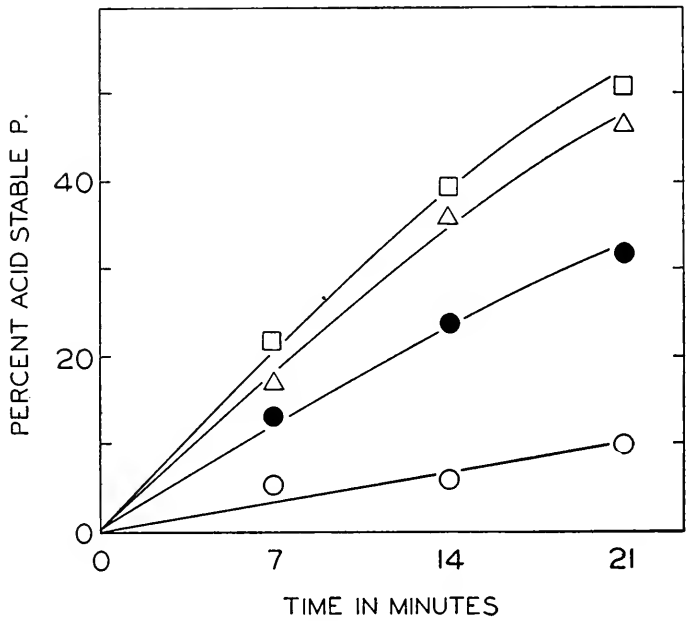

Figure 4. *Phosphoribomutase activity in the presence of different amounts of glucose-*1,6*-diphosphate.*

Ribose-1-phosphate, 3×10^{-3} M; magnesium sulphate, 10^{-3} M; trihydrochloric acid buffer, 2×10^{-2} M, pH 7·3; 8-hydroxyquinoline, 10^{-3} M; muscle enzyme, 60μg.; protein per ml.; glucose-1,6-diphosphate, synthetic sample. O control; ● glucose-1,6-diphosphate 2×10^{-6} M; △ glucose-1,6-diphosphate 3×10^{-5} M; □ glucose-1,6-diphosphate 8×10^{-6} M.

5-diphosphate, as was already suggested by Leder and Handler (1951). The nucleotide formation should then proceed as follows:

Ribose-1,5-diphosphate + base ⇋ nucleoside + orthophosphate.

The first indication of the existence of this di-ester was obtained from experiments on the enzymatic conversion of ribose-1-phosphate to ribose-5-phosphate (Klenow, 1953). This reaction is analogous to the phosphoglucomutase reaction which was shown by Cardini *et al.* (1949) to require glucose-1,6-diphosphate as a coenzyme. The mechanism of this reaction was found (Sutherland *et al.*, 1949) to be the transfer of the 1-phosphate of the coenzyme to the six-position of glucose-1-phosphate, whereby a new molecule of coenzyme and the reaction product, glucose-6-phosphate, are formed. Therefore the possibility existed that the phosphoribomutase reaction proceeded in a similar way, i.e. that it required ribose-1,5-diphosphate as a coenzyme.

During our study of the phosphoribomutase we found (Klenow, 1953) that the ratio between the activity of this enzyme in muscle extract and that of the phosphoglucomutase was altered only slightly during preparation of the latter as a crystalline enzyme (Najjar, 1948). Furthermore, it was found that the ribomutase reaction under certain conditions could be activated by glucose-1,6-diphosphate (see Figure 4). This suggested that the phosphoglucomutase could catalyse the transfer of a phosphate of glucose-1,6-diphosphate to ribose-1-phosphate, whereby a ribose-1,-5-diphosphate might be formed. This presumed that ribose-1,5-diphosphate might then function as a coenzyme for the phosphoribomutase reaction. Further evidence for the reaction

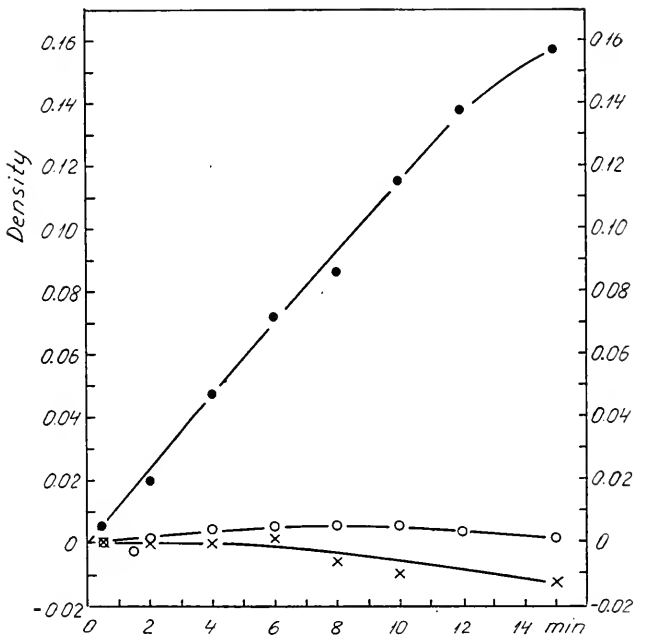

Figure 5. Formation of glucose-6-phosphate from ribose-1-phosphate and glucose-1,6-diphosphate.

Glucose-1,6-diphosphate: 6×10^{-5} M; ribose-1-phosphate: 3×10^{-4} M; triphosphopyridine nucleotide: $1{\cdot}2 \times 10^{-4}$ M; magnesium chloride: 2×10^{-3} M; glycyl-glycine cysteine buffer pH 7·2: 1×10^{-2} M; crystalline phosphoglucomutase: 0·015 mg. protein per ml.; *Zwischenferment:* 0·5 mg. protein per ml. ●complete ○ control without ribose-1-phosphate; × control without glucose-1,6-diphosphate. The reaction is measured in a spectrophotometer at 340 mμ.

between ribose-1-phosphate and glucose-1,6-diphosphate was obtained with glucose-6-phosphate dehydrogenase (*Zwischenferment*) and triphosphopyridine nucleotide. With this system it could be demonstrated that glucose-6-phosphate is formed from glucose-1,6-diphosphate in the presence of ribose-1-phosphate and phosphoglucomutase (Klenow and Emberland, 1954) (see Figure 5). In the same system it could furthermore be shown that not only ribose-1-phosphate, but also deoxyribose-1-phosphate and galactose-1-phosphate can serve as acceptors of a phosphate from glucose-1,6-diphosphate (Klenow, 1953). From incubation mixtures of ribose-1-

phosphate, glucose-1,6-diphosphate, and phosphoglucomutase a ribose phosphate has been isolated, the properties of which suggest it to be ribose-1,5-diphosphate.

The final proof that this compound is an intermediate in mono-nucleotide formation is, however, still lacking. Most recently a completely new type of pentose phosphate ester, important for these systems, has been isolated. These important findings were obtained from experiments on 6-carboxy-uracil, also called orotic acid, which is known to be the precursor of the uracil of nucleic acids. Kornberg (1954) found that orotic acid could be incorporated into a nucleotide by an enzyme system which, as in the foregoing cases, involved an activation of ribose-5-phosphate by adenosine triphosphate; he identified the activated form as 5-phospho-ribosyl-1-pyrophosphate. Whether this reaction proceeds in one step, i.e. consisting of a transfer of pyrophosphate from adenosine triphosphate to ribose-5-phosphate, or in two steps having ribose-1,5-diphosphate as an intermediate, still has to be seen.

With the 5-phospho-ribosyl-1-pyrophosphate the formation of the 5′-mononucleotide of adenine has been demonstrated to proceed as follows:

5-phospho-ribosyl-1-pyrophosphate + adenine $\leftrightharpoons$ 5′-adenylate + pyrophosphate.

Likewise orotic acid gave rise to orotodylic acid with the same reaction mechanism. The enzymes responsible for these reactions have been found in pigeon-liver acetone powder and in yeast. Thus this interesting new reaction for nucleotide formation is a reversible pyrophosphorolytic cleavage of the 5′-nucleotides. The establishment of this reaction might very well lead to the explanation of the enzyme reaction responsible for the synthesis of the imidazole and the pyrimidine rings of the purines. It has been found that in the case of inosinic acid the purine synthesis is completed only after introduction of ribose phosphate into the precursors (Greenberg, 1953). The formation of the nucleotides of these precursors might occur through Kornberg's new ribose phosphate ester as intermediate. In that case it might be possible to synthesize purine precursor ribotides enzymatically and with these to study the reactions which lead to completion of the purine rings.

Thus we have now accounted for some enzyme reactions by which ribose may be formed and for pathways for the formation of some ribosides and ribotides from ribose-phosphate esters and the appropriate purines and pyrimidines. How these nucleotides are linked together to form the nucleic acids is obviously a most appealing problem. No experimental evidence on this problem is yet in existence, but extremely stimulating theories have recently been advanced (Kalckar, 1953).

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Discussion

Chairman: C. H. Waddington

J. Brachet. Is there anything known about the intracellular distribution of the various enzymes involved in nucleotide synthesis?

H. Klenow. The enzyme system responsible for the incorporation of adenine into AMP in the presence of ribose-5-phosphate and ATP is present in the soluble part of pigeon-liver homogenate.

H. V. Brøndsted. Have you any indication that any of the B-vitamins enter into the synthesis of RNA? I am asking because we have shown that RNA accelerates regeneration in starved planarians, and so does riboflavin. The constituents of RNA given separately but in the right proportion act as a poison.

H. Klenow. It is known from the work of Greenberg and of Buchanan that citrovorum factor is significant for the formation of the purine part of inosinic acid. This acid can apparently be formed from 4-amino-5-imidazole-carboxamide ribotide and formic acid. This incorporation of formic acid, which appears in $C_{(2)}$ of the purine ring, seems to require citrovorum factor as a coenzyme.

Deoxynucleic acid in some gametes and embryos

by

E. HOFF-JØRGENSEN

Biokemisk Institut, Kobenhavns Universitet

THE sensitivity and specificity of the known chemical methods for the determination of DNA seem to be insufficient for the estimation of the minute concentration of DNA present in eggs and in embryos during the early stages of development. A microbiological assay method, which is very sensitive and highly specific, has been used in the investigations reported in this paper.

ASSAY METHOD

Principle. The lactic acid bacterium *Thermobacterium acidophilus* R 26 Orla Jensen (ATCC 11506) requires a deoxynucleoside as an essential growth factor. Neither vitamin B_{12} nor any other of many substances tested can replace the requirement for a deoxynucleoside. This organism therefore can be used as a test organism for microbiological assays of deoxynucleosides and also of DNA after depolymerization of the DNA. (Hoff-Jørgensen, 1952).

Stock cultures are maintained in the following medium by weekly transfer: 0·1 g. of cysteine and 0·5 g. of yeast extract (Difco) are dissolved in 100 ml. skimmed milk, at pH 6·8. The milk medium is dispensed in 2 ml. quantities to test-tubes (100 × 10 mm.). About 0·1 g. of $CaCO_3$ is added to each tube. The tubes are plugged with cotton, autoclaved at 120° C. for 10 min., inoculated with a wire loop, incubated for 24 hr. at 37° C., and stored in a refrigerator.

Inoculum medium. 50 ml. of the double-strength basal medium are mixed with 50 ml. of water. The minimum amount of peptone (e.g. about 5 mg. Difco per ml.) which gives maximum growth is added. The medium is dispensed in 5 ml. quantities to 15 ml. centrifuge tubes, each containing a glass bead. The tubes are plugged with cotton, autoclaved at 120° C. for 10 min., and stored in a refrigerator. Fresh inoculum medium is prepared every month.

Inoculum. A small loopful of the stock milk culture is transferred to a tube containing 5 ml. of the inoculum medium. After incubation at 37° C. for 20–24 hr., the cells are centrifuged, washed once with 10 ml. of sterile saline, and resuspended in 10 ml. of sterile saline. One small drop of this suspension is used to inoculate each assay tube.

Standard. Stock solution: 10^{-4} g. mol. of a deoxyriboside, e.g. 24·2 mg. of thymidine, is dissolved in 100 ml. of 25 per cent. ethanol. This solution is stable for at least one year.

Working standard: 5×10^{-9} g. mol. of deoxyriboside per ml. To prepare this, 50 μl. of the stock solution is diluted to 10 ml. with water.

Basal medium, double strength (100 ml.):

HCl-hydrolysed casein solution	30 ml.
Papain-hydrolysed casein solution	10 ,,
Salt A	5 ,,
Salt D	1 ,,
Tween 80	1 ,,
Cytidylic acid solution	1 ,,
Potassium acetate solution	5 ,,
Thioglycolic acid solution	1 ,,
Adenine-guanine-thymine solution	1 ,,
Vitamin solution	1 ,,
Glucose	3 g.
DL-Tryptophane	20 mg.
L-Cysteine	20 ,,

Dissolve the glucose, tryptophane and cysteine in the previously mixed solutions, adjust the pH to 6·7 with 1 N KOH, and add water to make 100 ml.

Prepare the various solutions as follows:

HCl-hydrolysed casein and papain-hydrolysed casein: as described by Hoff-Jørgensen, Moustgaard and Møller (1952).

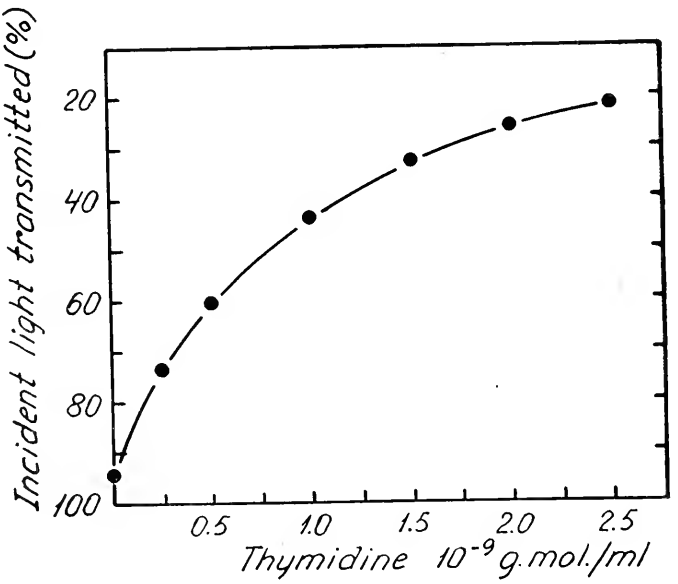

Figure 1. *Growth curve for* Tbm. acidophilus *R 26, 37° C., 24 hr.*

Salt A: dissolve 20 g. of monobasic potassium phosphate, KH_2PO_4, in water to make 100 ml.

Salt D: dissolve 0·3 g. of Mohr's salt ($Fe(NH_4)_2(SO_4)_2 . 6H_2O$), 0·2 g. of sodium chloride, 0·8 g. of manganese sulphate ($MnSO_4 . 4H_2O$), 4 g. of magnesium sulphate ($MgSO_4 . 7H_2O$), and 2 ml. of 1 N HCl in water to make 100 ml.

Tween 80 solution: dissolve 10 g. of Tween 80 (polyxyethylene sorbiton monooleate) in water to make 100 ml. Store in a refrigerator.

Cytidylic acid solution: dissolve 0·5 g. of cytidylic acid in water, adjust the pH to 7·0 with about 2 M sodium acetate solution, and add water to make 100 ml. Store in a refrigerator.

Potassium acetate solution: dissolve 500 g. of potassium acetate in water to make 1,000 ml.

Adenine-guanine-thymine solution: dissolve 0·2 g. each of adenine sulphate, guanine hydrochloride, and thymine with the aid of heat in 10 ml. of 2 N HCl. Add water to 100 ml.

Thioglycolic acid solution: dissolve 1 g. of thioglycolic acid in water to make 100 ml.

Vitamin solution: dissolve 0·5 mg. of folic acid and 5 mg. each of *p*-aminobenzoic acid, riboflavin, nicotinic acid and calcium pantothenate in 50 ml. of water. Store under a preservative in a refrigerator. Prepare a fresh solution every month.

Assay procedure

The assay is carried out in lipless *uniform* test-tubes (100 × 8 mm. i.d.). To each series of tubes the standard vitamin solution is added in the following amounts: 0·0, 0·1, 0·2, 0·4, 0·6, 0·8 and 1·0 ml. each with an error of not more than 2 per cent. Each level is set up in duplicate. The extract of the sample to be assayed is similarly added to a series of tubes in the following amounts: 0·2, 0·4, 0·6 and 0·8 ml., also in duplicate. All tubes are diluted to 1·0 ml. with distilled water and 1·0 ml. of the basal medium is added. The tubes are shaken, covered with glass or aluminium caps, autoclaved at 120° C. for 5 min., cooled to room temperature, and inoculated with one drop of the *immediately* previously prepared inoculum suspension. To two of the four tubes containing 0 ml. of standard no inoculum is added. These tubes are used as blanks in the turbidimetric determination of growth. All tubes are incubated at 37° C. for 24–36 hr.

Determination of response

The tubes are shaken and the turbidity is read in a photometer (e.g., Lumetron 402C, Photovolt Corporation, 95 Madison Avenue, New York, 16) at $\lambda = c.\ 650\ m\mu$. The microcuvettes are filled with a pipette and emptied with a piece of plastic tubing connected to a suction pump.

Calculation of results

A standard dose-response curve is prepared by plotting the average of the turbidity values found at each level of the deoxynucleoside standard against the amount of

deoxynucleoside present. The deoxynucleoside content of a sample is determined by interpolating the response to the known amount of the test solution onto this standard curve. The deoxynucleoside content per ml. of the test solution is now calculated for each of the duplicate sets of tubes, and the deoxynucleoside content of the sample is calculated from the average of the values.

Preparation of samples for assay

Deoxynucleosides: a solution containing about 3 mμmol. (or 0·5–1·0 μg.) deoxynucleoside per ml. is prepared in water, or in a not-more-than 0·05 M maleic acid buffer at pH 6·7.

Deoxynucleotides: incubation of a solution of deoxynucleotides with crude intestinal phosphatase (Schmidt and Thannhauser, 1943) is without effect on the response; it is therefore concluded that deoxynucleotides give the same response as deoxynucleosides on a molar basis.

Deoxynucleic acid. Pure DNA has a growth effect which is less than 1 per cent. of the effect of the deoxynucleosides present in the DNA. If, however, the DNA is depolymerized by deoxyribonuclease, (Kunitz, 1950) the growth response is equivalent to the effect of the calculated content of deoxyribosides in the DNA. Samples of bacteria, yeast or tissue may either be analysed in the wet state or after drying with acetone. For the analysis of bacteria and yeast, the cells should be disintegrated, e.g. in 'the tuning-fork disintegrator' (obtained from H. Mickle, Hampton, Middlesex, England). The sample containing at least 0·2 μg. P as DNAP is placed in a small test-tube. An exactly measured amount of 0·5 N NaOH solution (e.g. 0·5 ml.) is added, or if the sample is a solution, enough 1·0 N NaOH solution to make the final solution 0·5 N in NaOH. The tube is placed in a boiling water-bath for 15 min. During this time the tissue is disintegrated with a glass rod. After the incubation at 100° C. 5 vol. of a solution containing 0·06 g. mol. of maleic acid and 0·01 g. mol. of magnesium sulphate per l. are added for each vol. of 0·5 N sodium hydroxide used above. The pH of the mixture should now be 6·3–7·0. In order to depolymerize the DNA 0·1 ml. of a solution usually containing 100 μg. of crystalline deoxynuclease (Worthington, Biochemical Lab., Freehold, New Jersey, U.S.A.) is added and the mixture is incubated for 16–20 hr. at 37° C. For each new material assayed, the minimum amount of DNAase which gives maximum response should, however, be found by experiments. After incubation the mixture is diluted to contain about 3 mμ mol. deoxynucleoside per ml. and assayed (*one g. mol. deoxynucleoside* ~ 310 *g. DNA*).

Differentiation between purine and pyrimidine deoxynucleosides

As the pyrimidine deoxynucleosides are stable towards mild acid hydrolysis, whereas the purine deoxynucleosides are not, it is possible to distinguish between these two types of deoxynucleosides by assaying the depolymerized sample before and after boiling for 5 min. at pH 1. Before assaying the acid solution must be neutralized.

Specificity, sensitivity and accuracy

The method seems to be absolutely specific for the deoxyribonucleic linkage and allows the determinations of amounts greater than about 2 μg. of deoxynucleosides, deoxynucleotides or DNA with a standard deviation of about 5 per cent.

DNA IN GAMETES AND EMBRYOS OF *PARACENTROTUS LIVIDUS*

Material. The work was carried out at the Stazione Zoologica, Naples. To 600 ml. of an egg suspension containing about 4×10^4 eggs per ml. in sea water there was added 0·5 ml. of a sperm suspension containing about 10 sperms per egg. The egg suspension was placed at 20° C. and continuously mixed by a slow stirrer.

To fix the fertilized eggs or embryos for microscopic examination 9 ml. of the suspension were withdrawn and added to one ml. of 40 per cent. formalin. For the DNA determination 40 ml. of the suspension were withdrawn, cooled in ice water and centrifuged at low speed. The embryos were suspended in 40 ml. of acid sea water at pH 3·6 to remove the jelly capsule and adhering sperms, and again centrifuged. The washing with acid sea water was repeated once and followed by one washing with 40 ml. of distilled water to remove salts. The embryos were washed twice with acetone and once with ether and then dried in a vacuum desiccator over sulphuric acid. Washing and centrifuging were performed at 0° C. with precooled fluids. The treatment described above is without effect on the DNA content of the eggs.

Results

(1) DNA in sperm and unfertilized eggs:

(*a*) sperm

20 ml. sperm suspension $\sim$ 60 mg. dry matter

1 μl ,, ,, $\sim 0{\cdot}51 \times 10^6$ sperm

1 mg. dry matter $\sim$ 390 mμmol. deoxyriboside

per sperm: $$\frac{390 \times 60 \times 0{\cdot}310}{0{\cdot}51 \times 10^6 \times 20 \times 10^3} = 0{\cdot}71 \times 10^{-6}\ \mu\text{g. DNA}$$

(*b*) eggs

40 ml. egg suspension $\sim$ 70 mg. dry matter

1 ml. ,, ,, $\sim 4{\cdot}4 \times 10^4$ eggs

1 mg. dry matter $\sim 1{\cdot}35$ mμmol. deoxyriboside

per egg: $$\frac{1{\cdot}35 \times 70 \times 0{\cdot}310}{4{\cdot}4 \times 10^4 \times 40} = 16{\cdot}6 \times 10^{-6}\ \mu\text{g. DNA}$$

$$\frac{\text{DNA per egg}}{\text{DNA per sperm}} = \frac{16{\cdot}6}{0{\cdot}71} \sim 23$$

Elson and Chargaff (1952), using a microbiological assay of thymine, found about 25×10^{-6} μg. DNA per egg and $1{\cdot}0 \times 10^{-6}$ μg. DNA per sperm.

(2) DNA in embryos during the early stages of development:

hr. after fertilization		-1/2	3/4	1 1/2	3	4 1/2	6	7 1/2
number of cells per embryo	1	100	100	60	31	15	15	15
	2	–	–	40	10	2	1	–
	4	–	–	–	46	22	–	–
	8	–	–	–	13	41	–	–
	16	–	–	–	–	20	61	27
	>16	–	–	–	–	–	23	58
10^{-6} µg DNA per embryo		17.5	18.3	17.9	18.0	57.5	175	435

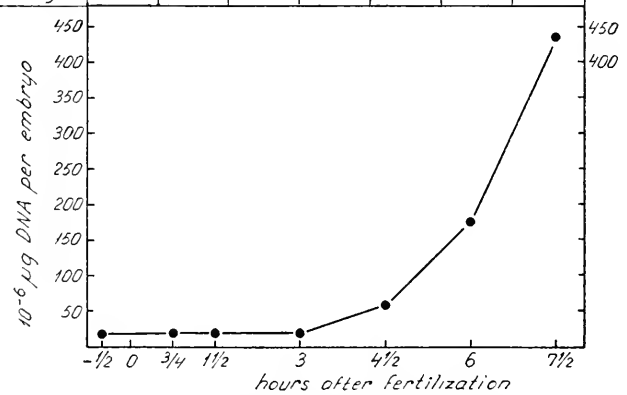

Figure 2. Table: Percentage of embryos (Paracentrotus lividus) *at different stages of development. Graph: Content of DNA per embryo or egg.*

Figure 2 shows that the content of DNA in the embryo is the same as in the unfertilized eggs until the 16-cell stage.

DNA IN EGGS, SPERM AND EMBRYOS OF *RANA TEMPORARIA*

Material. The eggs were fertilized as described by Rugh (1948). The jelly was removed with scissors. The eggs and embryos were dried with acetone and ether. In sperm DNA was determined without drying.

Results

(1) DNA in sperm and unfertilized eggs:

(*a*) sperm

4 ml. sperm suspension $\sim 1{\cdot}4 \times 10^6$ sperm $\sim 12{\cdot}6$ µg. DNA
per sperm: $8{\cdot}6 \times 10^{-6}$ µg. DNA

(*b*) unfertilized eggs

25 eggs $\sim 1{\cdot}73$ µg. DNA
25 ,, $\sim 1{\cdot}91$,, ,,
25 ,, $\sim 1{\cdot}82$,, ,,

average per egg: $7{\cdot}3 \times 10^{-2}$ μg. DNA

$$\frac{\text{DNA per egg}}{\text{DNA per sperm}} = \frac{7{\cdot}3 \times 10^{-2}}{8{\cdot}6 \times 10^{-6}} = 8{\cdot}5 \times 10^{3}$$

The value found for the DNA content per sperm seems high, but it agrees well with the finding by Mirsky and Ris (1949), that erythrocytes of the frog contain $15{\cdot}0 \times 10^{-6}$ μg. DNA per cell and hepatic tissue $15{\cdot}7 \times 10^{-6}$ μg. DNA per cell. If we take the average value $15{\cdot}35 \times 10^{-6}$ μg. as representing the DNA content of the diploid cells of the frog we get:

$$\frac{\text{DNA per egg}}{\text{DNA per cell}} = \frac{7{\cdot}3 \times 10^{-2}}{15{\cdot}35 \times 10^{-6}} = 4{\cdot}75 \times 10^{3}$$

which means that the egg contains enough DNA for about 5000 cells.

(2) DNA in embryos during the early stages of development (13°–17° C.):

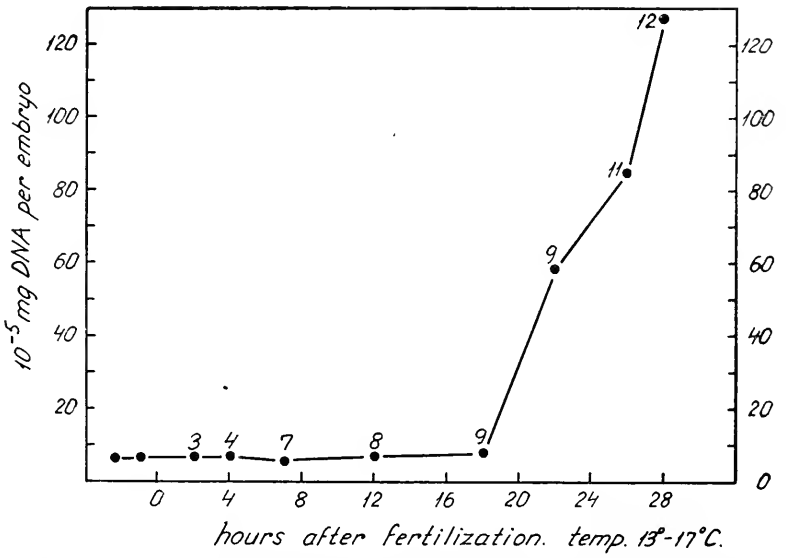

Figure 3. *Content of DNA in eggs and embryos of* Rana temporaria. *The figures above the curve indicate Shumway's stages of development.*

Figure 3 shows that the content of DNA in the embryo is the same as in the unfertilized egg until 18 hours after fertilization (Shumway's stage 9). At that stage a rapid synthesis of DNA begins. 5,000 cells at that time would correspond to an average generation time of about $1\frac{1}{2}$ hours.

DNA IN EGGS AND EMBRYOS OF THE DOMESTIC FOWL

Material. Fertilized eggs were incubated at 38° C. and 70 per cent. humidity. 2 × 2 eggs were taken out daily at the same time of day and treated as follows: 2 whole eggs (white, yolk and embryo) were treated in a blender with 500 ml. of

acetone and 250 ml. of ether for 10 min. at slow speed. After standing for 10 min. the acetone-ether was withdrawn and 300 ml. of ether added. After stirring for 5 min. the suspension was filtered, washed with ether and dried in a desiccator over sulphuric acid.

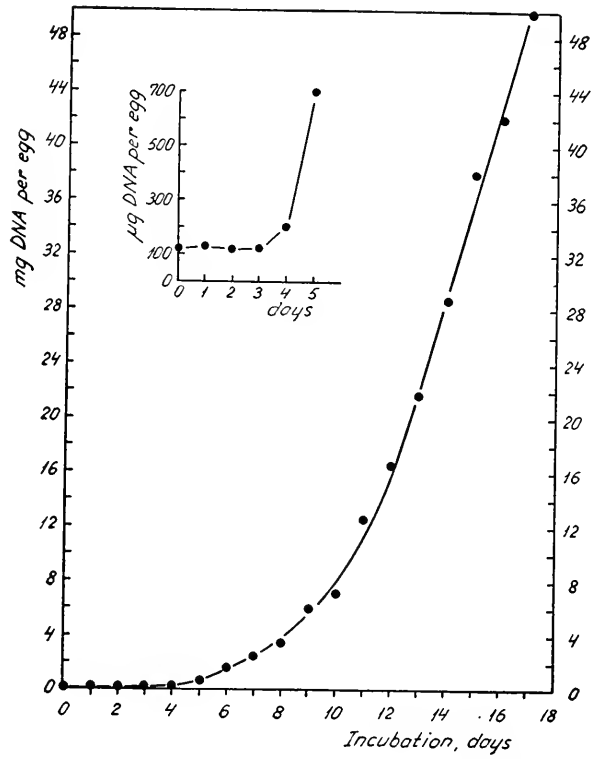

Figure 4. Content of DNA in eggs of the domestic fowl during development of the embryo.

Figure 4 shows that the content of DNA is the same until 3 days after incubation.

The average of 8 determinations of the content of DNA in unfertilized eggs (50–55 g.) was 118 µg. per egg with a standard deviation of 12 µg. Mirsky and Ris (1949) found $2{\cdot}34 \times 10^{-6}$ µg. DNA in erythrocytes and $2{\cdot}39 \times 10^{-6}$ µg. DNA in hepatic tissue per cell of the domestic fowl. If we take $2{\cdot}37 \times 10^{-6}$ µg. DNA as representing the DNA content of the diploid cells of the domestic fowl we get:

$$\frac{\text{DNA per egg}}{\text{DNA per cell}} = \frac{118}{2{\cdot}37}\,10^6 \sim 5 \times 10^7$$

indicating that the egg contains enough DNA for 5×10^7 diploid cells.

DISCUSSION

Hoff-Jørgensen and Zeuthen (1952) showed that in the egg of the frog most of the DNA must be located in the cytoplasm and made available for the formation of new

cells during the first stages of embryonic development. The egg of the sea-urchin *Paracentrotus lividus* is much smaller than the egg of the frog and contains correspondingly less DNA, namely only enough DNA for about 16 new diploid cells, in accordance with the finding that the synthesis of DNA begins at that stage of development.

The egg of the frog *Rana platyrrhina* contains about 10,000 times as much DNA as the sperm and about 5,000 times as much DNA as the diploid cells. It seems reasonable that the number of cells in the embryo at Shumway's stage 9, when DNA synthesis begins, is a few thousands; Bragg (1938) found about 10,000 cells at gastrulation in *Bufo cognatus*. The hen's egg contains enough DNA for about 5×10^7 diploid cells, and synthesis of DNA begins after 3 days of incubation, indicating that a 3-days old embryo contains about 5×10^7 cells. This number of cells presupposes an average doubling time of about three hours during the first three days of development, as 5×10^7 amounts to about 2^{25}.

Villee *et al.* (1949) found that inorganic ^{32}P is incorporated in the DNA of the sea-urchin's egg even during the first hours after fertilization. This indicates that before the increase in DNA begins the phosphate bonds of the cytoplasmic DNA are hydrolysed and the deoxynucleosides used in synthesis of specific nuclear DNA in the new cells. Two types of conversions of one deoxynucleoside to another are known. One is catalysed by a mammalian liver enzyme (Friedkin and Kalckar, 1950):
Deoxyribose-1-R + $H_3PO_4 \leftrightharpoons$ Deoxyribose-1-phosphoric acid + R; Deoxyribose-1-phosphoric acid + $R_1 \leftrightharpoons$ Deoxyribose-1-R_1 + H_3PO_4; the other is catalysed by extracts of some micro-organisms (McNutt, 1952):
Deoxyribose-1-R + $R_1 \leftrightharpoons$ Deoxyribose-1-R_1 + R where R and R_1 represent purines or pyrimidines.

SUMMARY

(1) A microbiological method, which allows the determination of a few μg. DNA is described.
(2) Using this method the following values for the DNA content of eggs and sperm have been found. In *Paracentrotus lividus*: $16{\cdot}6 \times 10^{-6}$ μg./egg; $0{\cdot}71 \times 10^{-6}$ μg./sperm. In *Rana platyrrhina*: $7{\cdot}3 \times 10^{-2}$ μg./egg; $8{\cdot}6 \times 10^{-6}$ μg./sperm. In the domestic fowl: 118 μg./egg.
(3) In the same species the DNA content in the embryos has been followed during development. An increase in the DNA content begins in the sea-urchin embryo at the 16-cell stage, in the frog embryo at about the 5000-cell stage, and in the hen embryo probably at about the 5×10^7-cell stage.

The author is indebted to Statens Almindelige Videnskabsfond for a grant and to Dr. R. Dohrn, Stazione Zoologica, Naples for help and hospitality. Grateful acknowledgement is made for many helpful suggestions and interesting discussions to Dr. E. Zeuthen of Copenhagen and Dr. A. Monroy of Palermo. Dr. Scherbaum of Copenhagen kindly made the microscopic examinations of the eggs and sperm.

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Discussion

Chairman: C. H. Waddington

J. Brachet. The estimation of DNA in the cytoplasm of eggs depends largely on the specificity of the method used. In the case of the sea-urchin and the frog, the more specific the method, the less is the DNA found in the egg. Using Ceriotti's method, which certainly is less specific than Dr. Hoff-Jørgensen's, I found about four times as much DNA in frog eggs as he did. However, even so, a definite synthesis of DNA was found to occur during cleavage. To explain these discrepancies other methods will have to be tried.

I am rather surprised at the conclusion that there is no DNA synthesis in chick embryos until after the third day of incubation, as the embryos at that stage have already undergone considerable morphogenesis. Is it possible that the yolk contains substances interfering with Dr. Hoff-Jørgensen's method? The material giving a positive Ceriotti reaction for desoxypentose is also in the yolk, but it is hard to see how desoxyribonucleosides could be released from yolk platelets, without digestion of the latter, in such a harmonious way that the liberated nucleosides would exactly match the requirement for nuclear multiplication. If yolk constituents interfered

with Dr. Hoff-Jørgensen's method, this would explain the much greater quantity of DNA in a hen's egg than in a sea-urchin egg. It would be interesting to study separately the DNA content of the embryos and of the yolk, and also to study the DNA content of embryos grown on Spratt's synthetic medium.

E. Hoff-Jørgensen. It is unlikely that other substances are responsible for the growth of the test organism. The exact amount of desoxyriboside found by the assay method can be recovered as DNA from the cells of the test organism. Also the hypothetical substance supposed to interfere with the test would have to be formed as a result of treatment with the highly specific crystalline DNA-ase; little or no growth factor is found without this treatment.

C. H. Waddington. It seems peculiar that the egg should not perform any synthesis of DNA until all its reserves are completely exhausted, and should then immediately start to synthesize at full speed. Moreover, at the time when synthesis begins in the chick embryo, the centre of the yolk is still a long distance away from the nearest cells (those of the yolk-sac). Is it possible that there is a continuous destruction of DNA, and that the synthesis at first balances this but that, eventually, as the number of nuclei increases, synthesis greatly surpasses breakdown?

E. Hoff-Jørgensen. We are determining the linkage between desoxyribose and a base, and all we can say is that during the first three days of development the number of these linkages is constant.

J. E. Harris. If one is prepared to admit that the bacterium cannot synthesize DNA, it is not unreasonable to suggest that the early embryo may not be able to do so either.

O. Maaløe. A breakdown of DNA to balance synthesis would have to be taken all the way down—beyond the nucleoside stage—if the degradation products are not to be detected by Dr. Hoff-Jørgensen's method.

C. H. Waddington. It would be interesting to try diploid or tetraploid frog embryos, as these would be likely to contain a different amount of DNA per cell.

J. Brachet. I have investigated haploid frog embryos produced by irradiation of the sperm with ultra-violet light. These embryos contain more cells than diploid embryos. There is less DNA in early cleavage stages; the DNA content catches up by the late blastula stage; but after gastrulation the haploid embryo lags behind once more in DNA content.

M. M. Swann. Early embryos relying on a store of DNA might be immune to inhibitors of DNA synthesis, such as aminopterin. Have any been tried?

E. Hoff-Jørgensen. The inhibitors tried—acting against thiamine, riboflavin, or folic acid—had no visible effect on the embryos. It is of course possible they they do not penetrate the egg membrane.

E. N. Willmer. Embryo extract stimulates the synthesis of nucleoproteins in tissue cultures of chick fibroblasts. One of the immediate effects of the addition of embryo extract is to increase the glucose uptake by the cells. It is therefore interesting to observe, as I did many years ago, that the sea-urchin egg does not pick up glucose

during the first day, but picks it up actively on the second day, by which time the synthesis of nucleoprotein is presumably going on.

E. Zeuthen. I should like to call attention to a paper by Blanchard (1935, *J. biol. Chem.* **108**, 251) who isolated 1·08 g. deoxynucleic acid from 4,820 g. of (wet) *Arbacia* eggs. The substance yielded negative biuret reaction, negative tests for pentose, and a positive Feulgen reaction. It contained 16·35 per cent. N and 10·13 per cent. P. Upon hydrolysis with 5 per cent. H_2SO_4 it yielded 11·4 per cent. guanine and 9·87 per cent. adenine, all values close to what is reported for DNA from other sources. RNA was demonstrated in amounts about equal to DNA.

Hoff-Jørgensen (for unfertilized *Arbacia lixula* eggs) finds 0·65 per mil. of the dry matter to be DNA. This would seem to compare reasonably well with Blanchard's value 0·23 per mil. for wet *Arbacia punctulata* eggs. The important fact remains that already in 1935 DNA seems to have been isolated from unfertilized sea-urchin eggs in yields which we can now see indicate that the egg holds far more DNA than the spermatozoon.

The excess DNA of the egg may be either in the nucleus or in the cytoplasm. With special regard to the sea-urchin egg I have (*Pubbl. Staz. zool. Napoli* **23**, suppl. 1951) suggested the latter possibility as the only one that would bring into harmony the very different results obtained for the whole egg and (cf. Lison and Pasteels, 1951, *Arch. Biol.* **62**, 1) for the nucleus alone.

A recent finding by Agrell (1953, *Ark. Zool.*) that the basophilia of the cytoplasm decreases in early cleavage can be taken as further support of the idea that nucleic acids move from the cytoplasm to the new nuclei as they form. I do think, therefore, that several lines of evidence available for the sea-urchin egg support the conclusion derived with Hoff-Jørgensen's method, that the cytoplasm of mature eggs stores DNA in amounts which—roughly—are in proportion to the egg size.

Nuclear control of enzymatic activities

by

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INTRODUCTION

WHILE the importance of the cell nucleus in heredity is a well-established fact, its general significance for the life of the cell still remains mysterious. Since the famous experiments on merotomy in Protozoa by Verworn (1881), Balbiani (1889) and their successors (for recent contributions to the problem, see Weisz (1948) and Tartar (1953)), it has been known that an enucleate piece of an unicellular organism is usually unable to regenerate; however, the important experiments of Hämmerling (1934) on the giant unicellular alga *Acetabularia* have clearly shown that, provided light is supplied, a non-nucleated fragment is capable not only of growth but even of real morphogenesis.

Three main hypotheses have been put forward to account for these experimental facts: (1) the nucleus is the main centre of cellular respiration (Loeb, 1899); (2) the nucleus is a storehouse of enzymes or of substances which can activate enzymes (E. B. Wilson, 1925); (3) the nucleus is the main centre of protein synthesis (Caspersson, 1941). These three theories will be discussed critically on the basis of experiments performed in this laboratory on unicellular organisms (*Amoeba proteus* and the alga *Acetabularia mediterranea*).

However, a few words should be said first about the results obtained by the many biochemists who have been working on homogenates: it is known, from the experiments of Claude (1943), that it is possible to isolate, by progressive centrifugation of extracts of crushed cells, various fractions which respectively consist of nuclei, mitochondria and microsomes (small granules). It is now a well-established fact that the mitochondria contain the vast majority of the oxidative enzymes and that they can oxidize both carbohydrates and fatty acids completely; furthermore, if they are supplemented with the necessary soluble coenzymes, they are even able to couple these oxidations with phosphorylations, i.e. to synthesize the energy-rich phosphate bonds characteristic of adenosinetriphosphoric acid (ATP).

The role of the microsomes has long been more mysterious; as they contain a large proportion of ribonucleic acid (RNA) and as this substance seems to play a part in protein synthesis (Brachet, 1941; Caspersson, 1941) it has been suggested that the microsomes are important agents in protein synthesis (Brachet and Jeener, 1944; Brachet, 1945). Such a suggestion has recently found very strong support in experiments on the incorporation of labelled amino-acids into proteins by various cell fractions (Hultin, 1950 *a*, *b*; Stern and Mirsky, 1952; Siekewitz, 1952; Gale and Folkes, 1953, etc.): they clearly show that microsomes are more active in protein synthesis

than other cell fragments, and that the integrity of RNA is essential for this process, as digestion of RNA by the enzyme ribonuclease leads to a strongly decreased incorporation of the labelled amino-acids into the proteins of the homogenates.

As regards the nuclei, their function is also obscure: they are very poor in respiratory enzymes, but they probably contain the enzymes which synthesize the nucleotides. For instance, according to Hogeboom and Schneider (1952), synthesis of the very important diphosphopyridinenucleotide occurs entirely in the isolated nuclei; other enzymes concerned with nucleotide metabolism (nucleosidephosphorylase, adenosinedeaminase) have also been shown to be concentrated in the nuclei (Stern *et al.*, 1952). Metabolism of RNA, as studied with labelled isotopes (^{32}P, ^{14}C-orotic acid) is also much more active in the nuclei than in the cytoplasm (Marshak, 1948; Jeener and Szafarz, 1950; Barnum and Huseby, 1950; Hurlbert and Potter, 1952, etc.): it might very well be that, as suggested by Marshak (1948) and by Jeener and Szafarz (1950), the nuclear RNA is a precursor of the RNA in the microsomes. It looks thus as if the nuclei were especially concerned with the synthesis of nucleotides, whether in the form of respiratory coenzymes or of nucleic acids.

There is no doubt that the work done on the chemical composition of the various fractions of homogenates, which was originated by Claude (1943) and by Brachet and Jeener (1944), has great importance for an understanding of the role of the various cell constituents. However, this type of work is open to many sources of error which obviously restrict the meaning of the results obtained: for instance, one is never sure that the various particles which are collected by differential centrifugation of homogenized cells are really the ones which pre-existed in the intact, living cell. During homogenization and centrifugation, soluble enzymes can be adsorbed on the particles while, on the contrary, the cell granules may release enzymes which are normally bound to them. These uncertainties become apparent when it is realized that the chemical properties of the cell constituents, especially the nuclei, are not the same when the cells are crushed in different media. A further limitation of the homogenate technique, obvious to any biologist who has a morphological background, lies in the fact that it is hardly possible to get information on the interactions occurring between the different fractions: mixing in a test-tube nuclei and mitochondria and following some enzymatic reaction, for instance, is a valid experiment from a biochemical viewpoint; but it is a meaningless and a possibly misleading undertaking if what we want to know is the nature of the interaction occurring between the nucleus and the mitochondria in a normal living cell.

It is because these shortcomings of the homogenate technique are so obvious that some of my co-workers and myself decided to compare the biochemical changes occurring in nucleated and non-nucleated fragments of unicellular organisms (*Amoeba proteus* and *Acetabularia mediterranea*): this is the only way to gain some understanding of the biochemical interactions between nucleus and cytoplasm in a living cell. This type of work is obviously limited by the sensitivity of the methods available, since relatively few organisms can be cut into nucleated and non-nucleated halves; but, thanks especially to Linderstrøm-Lang and Holter, many excellent ultramicromethods of analysis now exist. The present paper will deal with the main results of these experiments even though they are still, admittedly, quite fragmentary.

EXPERIMENTAL RESULTS

The nucleus and cellular oxidations

It is a well-known fact that non-nucleated halves of *Amoeba* soon round up and become incapable of normal feeding; in order to place nucleated and non-nucleated fragments in similar experimental conditions, it is necessary to leave both of them without external food supply during the whole experimental period. Under these conditions, nucleated halves survive three weeks and non-nucleated ones seldom live more than a fortnight.

Measurements of the oxygen consumption, made every other day on nucleated and non-nucleated halves of *Amoeba*, have shown that the respiration of both types of fragment remains identical for at least ten days, if the values are corrected for the differences in size of the pieces (Brachet, 1951).

Similar results have been obtained in the case of *Acetabularia mediterranea* (Chantrenne-Van Halteren and Brachet, 1952), where non-nucleated halves can survive up to five months: even three or four months after sectioning, non-nucleated pieces still respire at the same rate as the nucleated ones, provided the results are expressed in terms of dry weight.

These results are in good agreement with those we had obtained earlier (1939) when studying the oxygen consumption of isolated nuclei (germinal vesicles) from frog's oocytes; they also agree with the findings of Shapiro (1935) on the respiration of nucleated and non-nucleated halves of unfertilized sea-urchin eggs: in neither case does the cell nucleus play a leading role in cellular oxidations, as was believed by Loeb (1899).

When the oxygen consumption of cells which have been ultra-centrifuged in the living condition is studied, it is found that the main respiratory enzymes are actually bound to large cytoplasmic granules, comparable or identical with the mitochondria: this conclusion can be drawn from the experiments of Ballentine (1939) on unfertilized sea-urchin eggs, of Huff and Boell (1936) on *Ascaris* eggs, and of Chantrenne (1944) on frog's liver.

It is therefore obvious that the nucleus cannot be the main centre of cellular oxidations and that Loeb's theory is no longer tenable; work on intact cells or organisms entirely confirms the results obtained on homogenates in showing that large cytoplasmic granules, presumably the mitochondria, contain the more important respiratory enzymes. Furthermore, the work done on unicellular organisms clearly shows that these mitochondria are very largely independent of the presence or the absence of the nucleus for normal functioning.

Ribonucleic acid (RNA), proteins and glycogen

We have already seen that RNA is mostly found in the small cytoplasmic granules (microsomes), as well as in the nucleoli, and that this substance is supposed to play some important role in protein synthesis (Caspersson, 1941; Brachet, 1941).

In amoebae, both cytochemical techniques (Brachet, 1950) and quantitative micro-estimations (Linet and Brachet, 1951) have clearly shown that the removal of the nucleus is followed by a considerable drop in the RNA content of the cytoplasm: 3 days after sectioning, non-nucleated halves become poorer in RNA than the

nucleated ones; 10 days after the operation, the non-nucleated cytoplasm contains less than 30 per cent. of the initial RNA content.

It is worth mentioning that reserve foodstuffs—glycogen and lipids—behave very differently from RNA: non-nucleated halves utilize them to a small extent only during the 2–3 days which follow sectioning; they then become unable to utilize these reserve materials. In nucleated fragments, on the other hand, glycogen and lipids undergo a much larger decrease during the early days of fasting; their utilization becomes much slower afterwards, however. Total proteins also decrease faster during the initial days after sectioning than afterwards: the impressive loss of RNA in non-nucleated fragments thus occurs precisely when the utilization of carbohydrates, fats and proteins has practically stopped.

Since cytoplasmic RNA is accumulated in the microsomes, in amoebae as elsewhere, there is no doubt that these small particles depend much more for their maintenance on the presence of the nucleus than do the mitochondria. That such a conclusion is of general significance is shown by the fact that, during the ripening of mammalian red blood cells, the loss of the nucleus is followed by the disappearance of the basophilic cytoplasmic network, which is rich in RNA. Furthermore, preliminary experiments on non-nucleated *Acetabularia* fragments have shown that their RNA content is reduced by 50 per cent. after 2 weeks (Szafarz and Brachet, 1954).

The rapid disappearance of RNA from non-nucleated cells is obviously consistent with the view, expressed by Caspersson, (1941), Marshak (1948), and Jeener and Szafarz (1950), that nucleolar RNA might be a precursor of microsomal RNA. However, recent experiments on *Acetabularia* (Brachet and Szafarz, 1953; Szafarz and Brachet, 1954) in which labelled orotic acid was used as a precursor of RNA, have shown that normal metabolism and turnover of the cytoplasmic nucleic acid goes on for many weeks without a nucleus, at any rate in the presence of light.

Whether the strong decrease in RNA content of enucleated amoebae is followed by a reduction in protein synthesis is not yet known for certain: present work with autoradiographic techniques in this laboratory indicates that incorporation of labelled glycine in the proteins markedly drops when the RNA content of the non-nucleated fragments decreases. There is no doubt that, in the case of the reticulocytes, both the RNA content and the uptake of labelled amino-acids into the proteins go down in a parallel manner (Holloway and Ripley, 1952; Gavosto, Ficq and Errera, 1954; Gavosto and Rechenmann, 1954). In the growing starfish oocytes, elegant experiments by our co-worker A. Ficq (1953) have clearly shown that the incorporation of radioactive glycine into the proteins is especially active in the nucleolus and in the basophilic cytoplasm, both of which are very rich in RNA.

The problems raised by protein synthesis in non-nucleated pieces of *Acetabularia* will be discussed more fully later on; but it should immediately be said that, here again, the drop in RNA content and that in protein synthesis are parallel events.

Enzymatic composition of nucleated and non-nucleated fragments of amoebae

The decrease in the RNA content of non-nucleated halves of *Amoeba* certainly means that the microsomes are seriously affected when the nucleus is removed; it is unfortunately impossible as yet to say whether we are dealing with a complete destruction of some of the microsomes or with a mere alteration of their chemical

composition; work on homogenates, especially, is unreliable when one is using very small amounts of material, as is the case with fragments of *Amoeba*. It is to be hoped that improvements in the techniques of homogenization and centrifugation will make a quantitative investigation on homogenates of amoebae possible.

What is, however, feasible is a study of the enzymatic composition of nucleated and non-nucleated halves (Brachet, 1952*a*; Urbani, 1952*a*, 1952*b*): two of the enzymes studied so far, esterase and acid phosphatase, are present in large amounts in the microsomes, in the case of the mammals at any rate. Three other enzymes, adenosinetriphosphatase, amylase, and protease have been followed simultaneously: the first is bound mostly to mitochondria in rat liver, while the other two are present in the large granules of the amoebae (Holter and Pollock, 1952; Holter and Løvtrup, 1949). Finally, we studied also dipeptidase, as this enzyme, according to Holter and Løvtrop, (1949), is soluble and is thus apparently not bound to any type of cytoplasmic granules.

These experiments have yielded perfectly clear results: while the 'mitochondrial' enzymes (adenosinetriphosphatase, amylase and protease) are completely unaffected by the removal of the nucleus, even after 12 days, the 'soluble' dipeptidase behaves like total proteins: after an initial drop, the amount remains constant in non-nucleated halves. Finally, the enzymes which are presumably bound to the microsomes (acid phosphatase and esterase) behave exactly like RNA: only after the third day after bisection of the amoebae does their amount decrease in the non-nucleated half, but this drop is so pronounced that, on the twelfth day, only 20–30 per cent. of the initial amount is still left.

These findings make it likely that the control exerted by the nucleus on the various cell proteins largely depends on their intracellular distribution: negligible in the case of mitochondria, nuclear control is weak for soluble proteins and very important for microsomes, which probably disappear rather rapidly in the absence of the nucleus.

Such a view is rather different from the hypothesis of E. B. Wilson (1925): none of the enzymes we studied are accumulated in the nucleus and there is no evidence that they are synthesized in the nucleus itself. Nuclear control of enzymatic activities is obviously much more complex than was expected, as various proteins behave very differently after removal of the nucleus.

The nucleus and the production of coenzymes

It should first be emphasized that none of the enzymes we have studied are present in the nucleated fragments in much larger amount than in the non-nucleated half: none of them is therefore predominantly accumulated in the nucleus. We know, however, from work on homogenates that the enzymes which catalyse the synthesis of nucleotides, including various coenzymes, are on the contrary concentrated in the nuclei (Stern *et al.*, 1952; Hogeboom and Schneider, 1952).*

Technical difficulties have unfortunately prevented us so far from studying these interesting enzymes; but some of the results we have obtained on fragments of

* Recent unpublished experiments in this laboratory (E. Baltus) have shown that nucleoli isolated from starfish ovocytes are very rich in nucleosidephosphorylase, but contain little guanase or adenosinedeaminase. The enzyme which catalyses the synthesis of diphosphopyridinenucleotide is also accumulated in these nucleoli.

amoebae lend some support to the idea that the nucleus might play an important part in the production of coenzymes and perhaps regulate in this way the activity of the cytoplasmic enzymes.

We have seen earlier that non-nucleated fragments of *Amoeba proteus* retain a normal oxygen consumption for as long as ten days; however, a very striking and rapid change, first described by Mazia and Hirshfield (1950), and confirmed afterwards in this laboratory (Brachet, 1952), occurs when the penetration into the cell of radioactive phosphate is followed: the ^{32}P uptake is strongly reduced in the non-nucleated halves, only a few hours after the removal of the nucleus. Since oxidations and phosphate uptake are usually closely linked, we were led to believe (Brachet, 1951, 1952*a*) that enucleation interrupts the normal coupling between oxidations and phosphorylations, just as poisons like dinitrophenol, usnic acid, gramicidine, etc. do; if so, production by the nucleus of coenzymes necessary to couple oxidations with phosphorylations seemed a plausible explanation for the experimental results.

Later experiments (Brachet, 1952*b*) have shown, however, that such a simple hypothesis can hardly be correct, since the adenosinetriphosphate (ATP) content of nucleated and non-nucleated halves remains almost identical for a considerable length of time (twelve days): a drop in the ATP content of the non-nucleated halves was of course to be expected if removal of the nucleus really uncoupled phosphorylations from oxidations.

But very recently (Brachet, 1954) we have studied the ATP content of nucleated and non-nucleated halves when they are placed in anoxic conditions, which strongly affect the motility of the amoebae (Pantin, 1930; Kitching, 1939); it was found that, in the case of fragments from amoebae which had been sectioned for a few hours only, there is a 70 per cent. drop in the ATP level of the non-nucleated halves after 16 hours' anaerobiosis; this drop is only 15–20 per cent. in the case of the nucleated halves. When the fragments are 4–9 days old, the differences become less: we obtain a 50 per cent. drop in the case of the nucleated halves, as compared with 70 per cent. again in the non-nucleated ones.

These findings are probably correlated with the fact, already mentioned in this paper, that utilization of glycogen is much poorer in the non-nucleated than in the nucleated halves: during the initial 1–3 day period, the utilization of glycogen by the nucleated halves is faster than it is later on; in the non-nucleated fragments there is a restricted utilization of glycogen at the beginning (1–3 days), and it completely ceases thereafter. Preliminary cytochemical observations on the glycogen content (Bauer's method) of both types of fragment in anaerobiosis have shown that nucleated halves from well-fed amoebae (0–1 day after bisection) lose a very large proportion of their glycogen during the 16 hours' period of anaerobiosis; such striking changes do not occur in the non-nucleated halves of freshly bisected amoebae. Apparently, removal of the nucleus quickly and simultaneously impairs both glycogen breakdown and ability to keep ATP phosphorylated, under anoxic conditions.

These results strongly suggest that the nucleus controls certain steps of glycolysis, a conclusion which agrees with the observation of Stern and Mirsky (1952) that wheat germ nuclei are richer in several glycolytic enzymes than the cytoplasm; however, it was found (Brachet, 1954) that enolase, which according to Stern and

Mirsky (1952) is definitely accumulated in these nuclei, has an almost even distribution in both halves of *Amoeba*, even 11 days after bisection.

This negative result leads us to believe that the nucleus plays a part in the activation of glycolytic enzymes, by the production of the necessary coenzymes, rather than in their actual synthesis. Further work will of course be required to decide on which step the nucleus is acting; the fact that glycogen is involved and that phosphate penetration is reduced in non-nucleated halves seems to point towards the phosphorylase system, which requires for full activity—besides the protein—adenylic acid, –SH groups, and inorganic phosphate.

It is worth mentioning that decreased capacity to keep ATP in the phosphorylated form during anaerobiosis and reduced utilization of glycogen might very well prove to be biochemical abnormalities characteristic of any cell the nucleus of which is severely altered: these very same metabolic troubles have already been found in strongly lethal interspecific frog hybrids (Barth and Jaeger, 1947; Gregg, 1948), as well as in frog eggs fertilized with nitrogen-mustard-treated sperm (unpublished experiments).

PROTEIN SYNTHESIS IN THE ABSENCE OF THE NUCLEUS IN *ACETABULARIA*

The very important finding of Hämmerling (1934), that a non-nucleated piece of *Acetabularia* is capable of considerable growth and regeneration when light is supplied, has been confirmed and extended by our co-worker F. Vanderhaeghe (1952): her quantitative measurements of the dry weight and the total nitrogen content of nucleated and non-nucleated fragments have shown that growth and protein synthesis proceed at the same rate in both halves during 2–3 weeks; they then fall off considerably in the enucleate fragments.

This decrease in the potentiality for growth and regeneration after 2–3 weeks has also been shown in simple experiments by Brachet and Brygier (1953): if non-nucleated halves are placed in the light immediately after the bisection, 50 per cent. of them soon produce umbrellas; the same proportion of umbrellas is obtained even if the fragments have been left in the dark, where neither growth nor regeneration occurs, for 1 or 2 weeks prior to illumination; but when the non-nucleated halves are kept for 3 weeks in the dark before illumination, only 15 per cent. form umbrellas. Finally, when these non-nucleated pieces are left for 4 weeks in darkness, no umbrellas are produced in the light afterwards.

Biochemical experiments with $^{14}CO_2$ (Brachet and Chantrenne, 1951) have entirely confirmed these biological findings: incorporation of $^{14}CO_2$ in the proteins (carboxylic group of the amino-acids) is identical in both nucleated and non-nucleated fragments at the beginning, but it drops in the non-nucleated halves after 2–3 weeks and thereafter falls steadily. Incorporation of labelled CO_2 in the proteins of *Acetabularia* only occurs in the light and in the proteins of the chloroplasts (Brachet and Chantrenne, 1951); if glycine-^{14}C instead of $^{14}CO_2$ is used as a precursor, it is incorporated in the proteins in the dark as well as in the light, and the microsomes are now more active than the chloroplasts (Brachet and Brygier, 1953). The alga thus possesses two different mechanisms of protein synthesis, according to the experimental conditions; such a finding is not without interest since it is known from

the work of Beth (1953) that the stem of the alga can grow with a limited supply of light, while the formation of the umbrella definitely requires light of high intensity. The preferential utilization of one or other of the two mechanisms for protein synthesis might thus have far-reaching morphogenetic consequences.

Finally, it has also been shown (Brachet and Chantrenne, 1953; Chantrenne, Brachet and Brygier, 1953) that, in certain batches of algae, the non-nucleated fragments can react to the addition of 10^{-3} M H_2O_2 in the medium by increased catalase activity; whether we are dealing with a real adaptative enzymatic synthesis is not yet certain, but there is no doubt that after 2–3 weeks the capacity for increased catalase activity on the addition of H_2O_2 steadily decreases in the non-nucleated halves. Increased catalase activity, although quantitatively much reduced, is still found when 3-month-old nucleated pieces are placed in sea water containing H_2O_2.

These experiments show clearly that in *Acetabularia* the nuclear control of protein synthesis is a remote one; it is difficult to reconcile these findings with Caspersson's (1941) view that the nucleus is the main centre of protein synthesis. The experimental results we got on *Acetabularia* are in good agreement with those obtained with the basophilic non-nucleated red blood cells (reticulocytes): these also incorporate labelled amino-acids to a large extent into their proteins, for instance hemoglobin (London *et al.*, 1950; Holloway and Ripley, 1952; Chantrenne and Koritz, 1954; Nizet and Lambert, 1953; etc.). In both cases (Szafarz and Brachet, 1954, for *Acetabularia*; Holloway and Ripley, 1952, for reticulocytes) the decrease in protein synthesis and RNA content run parallel, in accordance with our hypothesis (1945) that microsomes play an essential part in protein synthesis.

It should, however, be pointed out that these results do not exclude the possibility that in the normal cell considerable protein synthesis may occur in the nucleus: as already pointed out earlier in this paper, recent observations by A. Ficq (1953, 1954) who used an autoradiographic method, have demonstrated considerable incorporation of radioactive glycine both in the proteins and in the ribonucleic acid of the nuclei in various embryonic and adult tissues. In the case of starfish oocytes, incorporation of glycine is very much higher in the nucleolus than in the nuclear sap or cytoplasm.

CONCLUSIONS

As most of the experimental results reported in this paper have already been thoroughly discussed, the conclusions are brief. It is now obvious that the nucleus is neither the centre of cellular oxidations nor a storehouse or building place for all enzymes; it might, however, be concerned with the synthesis of nucleotides, whether nucleic acids or coenzymes, and in this way might regulate many of the biochemical activities of the cell. To consider that the nucleus is the main centre of protein synthesis in the cell is also an exaggeration, since extensive building up of proteins can still go on in its absence; this does not mean that in a normal cell, especially a growing one, important protein syntheses cannot occur also in the nucleus, particularly in the nucleolus.

Our knowledge of the chemical changes undergone by the cell when its nucleus has been removed is still too incomplete for drawing far-reaching conclusions: but it

looks as if enucleation leads to a decrease in phosphorylation reactions, perhaps owing to the lack of some essential co-factors normally produced by the nucleus. These early biochemical abnormalities are quickly followed by a fall in the RNA content, obviously representing the alteration or the loss of some of the microsomes. The importance of the latter, and of the integrity of their RNA for protein synthesis, is now an established fact (Stern and Mirsky, 1952; Gale and Folkes, 1953); it is therefore not surprising to find a fall in the capacity of the enucleated cell for protein synthesis just at the time when the microsomes are injured. On the other hand, the activity of the mitochondria in cell respiration, and presumably of the chloroplasts in photosynthesis, is much less dependent on the presence of the nucleus.

Such a picture is still little more than a working hypothesis, and it is obvious that much more experimental work will be required before it can be taken as substantiated; but it is worth noting that, in a recent review on biochemical genetics, Bonner (1952) concludes that enzymatic synthesis occurs not in the genes themselves but in 'organized cytoplasmic particles'. Since microsomes are much more dependent than mitochondria on the presence of the nucleus, since they are rich in RNA and since they strongly incorporate amino-acids in their proteins it is likely that Bonner's cytoplasmic particles are nothing else but microsomes. It is a gratifying observation that cytochemistry, biochemistry and genetics, starting from very different viewpoints, all point in the same direction.

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Discussion

Chairman: E. Zeuthen

J. F. Danielli. Professor Brachet's results show that in enucleated fragments there is a decline in RNA content and a decline in protein sythesis. These results are compatible with the view that RNA is concerned in protein synthesis, or as I have suggested elsewhere, (1953, *Cytochemistry*, John Wiley, New York), with the view that RNA acts as a folding or trapping agent for protein. Is Professor Brachet aware of any evidence incompatible with the second suggestion? Dr. Yemm has already pointed out that plant virus nucleoprotein does not exchange amino-acids once it is combined with RNA. Another phenomenon which is explicable on this basis is the occurrence of protamine in sperm; being a highly basic protein it combines with nucleic acids and prevents protein synthesis, thus leading to sperm having little protein other than nucleoprotein.

J. Brachet. Our results are compatible with the view that RNA bound to the microsomes is important for protein synthesis, an opinion for which Mirsky's group has recently found fresh evidence. I think that they are compatible with Professor Danielli's hypothesis, without however giving it direct support.

G. Pontecorvo. What is the evidence for active synthesis of RNA within the nucleus, and for this RNA being the same as, or a precursor of, the cytoplasmic RNA?

J. Brachet. There is no doubt that although the decrease of RNA after removal of the nucleus is consistent with the idea that nuclear RNA might be a precursor of cytoplasmic RNA, it does not prove that relationship: the facts can easily be explained by other hypotheses. On the other hand it is now certain that in many types of cells the incorporation of labelled precursors of RNA (^{32}P, orotic acid) occurs very much faster in the nucleus than in the cytoplasm: this fact has been taken to show that nuclear RNA is a possible precursor of cytoplasmic RNA. However, a recent mathematical analysis of the data by Barnum and Huseby indicates that in cancer cells at any rate, nuclear RNA cannot be the precursor of cytoplasmic RNA. It thus seems that nuclear and cytoplasmic RNA are being synthesized independently, the former at a much faster rate.

E. W. Yemm. With regard to the possibility that nucleic acids may have a stabilizing action on proteins in the cell, the case of virus particles is relevant. It is well established that these are often nucleoproteins and there is evidence from the use of tracers that, once formed, they do not take part in replacement or exchange reactions to the same extent as normal cytoplasmic proteins. The relatively large amounts of nucleic acid associated with proteins in nuclear structures may be a significant factor favouring stability of the proteins at certain stages of development of the cells.

J. Brachet. Dr. Yemm's suggestion is very interesting; it is true that DNA and histone, in experiments with isotopes, also show an unusual stability. However, the other nuclear proteins are metabolically much more active.

R. J. Goldacre. Have you any views on the part played by the nucleus in the locomotion of *Amoeba*? In contrast to the long-term and indirect effects of enucleation which have just been described, the effect on the locomotion of amoebae is a rapid one, and the dramatic revival of active locomotion on replacement of the nucleus may take only a few minutes or even seconds. Have any chemical changes been correlated with the loss of active locomotion?

J. Brachet. I do not know what chemical change takes place. The decrease in the uptake of ^{32}P in non-nucleated halves of *Amoeba* discovered by Mazia also occurs very quickly after loss of the nucleus. I might also mention that non-nucleated halves stain uniformly with neutral red *in vivo*, whereas nucleated ones show the accumulation of the dye at the 'tail' described by Goldacre; furthermore, when a vitally stained *Amoeba* is cut in two, this accumulation of dye very quickly disappears from a non-nucleated half.

R. D. Keynes. Does the effect of enucleation on the uptake of ^{32}P follow or precede the effect on the ATP content? In other words, is the uptake reduced merely when the internal ATP falls, or is there a direct connexion between enucleation and uptake of phosphorus which is independent of the amount of ATP already in the cell?

J. Brachet. The decrease in uptake of ^{32}P in non-nucleated halves is the earliest chemical change so far discovered. The ATP content of non-nucleated halves does not decrease in the presence of air for as long as ten days. As a matter of fact the non-nucleated *Amoeba*, as well as the lethal frog's eggs, seem rather to have too high an ATP content: this might mean that they are unable to utilize ATP. The marked decrease in the ATP content of non-nucleated halves under anoxic conditions is also a very early phenomenon, possibly simultaneous with the reduction in uptake of ^{32}P.

E. Hoff-Jørgensen: Microbiological methods, which are useful for the estimation of coenzymes, might be used in the investigation of the effect of the nucleus on the synthesis of coenzymes.

The cell physiology of early development

by

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It is during embryonic development that the fundamental character of the cells composing the organism is formed. The cellular processes occurring at this time must be of a somewhat special and particularly interesting kind. Whereas adult cells synthesize some molecular species essentially similar to those which are already present, during embryonic development and differentiation new types of synthesis must come into operation. I wish in this paper to discuss what sort of picture our present theoretical outlook allows us to form of these processes. It is only in the light of some theoretical scheme that we can decide what are the most important questions to ask, and in what directions we should look for further insight into the fundamental nature of the mechanisms with which we have to deal.

It will probably be well to start by reminding ourselves of the general type of phenomenon with which we are confronted during differentiation. There are, of course, innumerable examples which we might take, but let us consider the developing tissue of the eye of a vertebrate such as the newt. By the time the fertilized egg has reached the stage where it is ready to gastrulate, the presumptive eye tissue is located in the roof of the blastocoel. The tissue is still capable of developing into a large number of different things. It can form any of the ectodermal derivatives, and it can also be converted into mesoderm and become any mesodermal type of tissue. It has, perhaps, already lost the capacity to form endodermal tissues. By the end of gastrulation the presumptive eye tissue is located in the neural plate. It is no longer capable of developing into any of the derivatives of the main axial mesoderm, or of the non-neural ectoderm. It is, however, beginning to acquire the capacity to develop without further stimulus from outside itself into relatively specific types of neural derivative. Within a fairly short time the range of possible types of tissue into which it can develop becomes more narrowly restricted, while at the same time the responses of the cells to external stimuli are reduced. Recent work (cf. Nieuwkoop and others, 1952) makes it probable that the tissue first acquires the capacity to develop into the various derivatives of the ectomesodermal neural crest, and shortly afterwards that to develop into anterior regions of the brain. At this stage it is still capable of responding to a trunk organiser, which can convert it from forebrain neural tissue into the neural system of the hindbrain or trunk. By the stage of the neural groove, however, the tissue has been rather firmly determined to develop into the eye. It can do so autonomously when isolated from the rest of the embryo and placed in a neutral medium. It has, however, still some flexibility, since a particular group of cells may form either part of the eye stalk, or of the retina, or of the tapetal layers of the eye cup. It is only some time later, after the optic vesicle has folded out from the brain

and the eye cup has been formed, that this flexibility disappears. The presumptive eye tissue is then said to be fully determined. For most other types of cells there is little evidence that such determination can later be reversed. In the particular case of the eye, however, we have the peculiar phenomenon of Wolffian regeneration of the lens, in which, after removal of the normal lens of a fully developed eye cup, some of the retinal cells lose their differentiation and eventually develop again in another direction to form a lens to replace that which is missing.

We see that the development of the eye takes place in a series of steps. In each step the developmental capacities of the cells are, in the first place, restricted, so that the later cells are not capable of so many different types of differentiation as the earlier ones; or perhaps, to be more cautious, we should put it that they are not so easily persuaded to undertake so many different types of differentiation as earlier ones are. But the steps are not purely restrictive, since at each successive stage the cells become less dependent on external influences for carrying out a full course of differentiation leading to an adult end-product. Finally we may note that even in cells which are at or near their final state of differentiation it may, under some circumstances, be possible to reverse the course of events and to cause the cells to differentiate again into a different type of end-product. The Wolffian regeneration of the lens is a striking example of this, and it seems probable that similar events occur in the regeneration of other organs in vertebrates.

We need now to try to form some idea of the physiological processes in the cells which may underlie this type of phenomenon. We must picture some sort of dynamic system which gradually alters in such a way that the types of change open to it become ever more restricted, while at the same time it passes through a series of stages of responsiveness to external influences, the range of external conditions to which it responds becoming less as time proceeds. These are the two phenomena known in embryological terms as 'increasing determination' accompanied by a progressive 'restriction of competence'.

What types of basic elements must be supposed to be involved in this dynamic system? There is no doubt that one category are the genes in the nucleus. The facts of genetics leave no doubt at all that these play a major role in determining the nature of the processes which will occur during differentiation and thus of the final end-product which will be produced. Again, experimental embryology has shown conclusively that in many eggs the cytoplasm is not the same in all regions of the egg, but is locally differentiated; and further that these local differences play a part in determining the type of differentiation which will occur in the cells formed out of them. We have to deal, therefore, with a dynamic system involving both nucleus and cytoplasm. Genetics has succeeded in analysing the constitution of the nucleus in considerable detail, but the analysis of the cytoplasm into unequivocally recognizable elements has been more difficult. There are, however, some categories which we may postulate with some confidence.

In the first place there must be what we may call the raw materials, that is to say, low-molecular-weight substances such as amino-acids, out of which the more complex cytoplasmic constituents, such as proteins, as well as the genes themselves, can be synthesized. A second category must be what we may call the final cell constituents. By this I mean the characteristic substances which endow the cell with its

particular histological character, for instance the myosin in a muscle cell, the secretory enzymes in a gland cell, the proteins which give rise to keratin in an epidermal or hair-forming cell, etc. Intermediate, as it were, between the raw materials and the final cellular products there must be a number of other categories. About these we know much less. There must, however, be substances which mediate the effects which the genes produce. We may tentatively consider a category of 'immediate gene products' given off by the genes and passing into the cytoplasm either at cell division when the nuclear membrane is destroyed, or perhaps continuously. These must interact with other substances already present in the cytoplasm. Possibly the immediate gene products control the whole progression from the cytoplasmic raw materials to the cellular final products, but it may be that the cytoplasm also contains other substances on the path between these two extremes not directly controlled by the immediate gene products.

Among the cytoplasmic constituents intermediate between the raw materials and the final products there may, indeed, be entities with properties of a somewhat gene-like nature. In recent years a great deal of study has been devoted to factors which may in general be termed 'plasmagenes', and extremely important advances in our knowledge of them have been made. The term 'plasmagene' covers a wide range of different types of entity. It has often been suggested, though in somewhat vague terms, that they may play an important part in cellular differentiation. It is worth discussing at some length the different sorts of plasmagenes whose existence has been demonstrated, and attempting to evaluate their possible role in embryonic development.

Broadly speaking, plasmagenes are revealed by two different types of evidence. On the one hand, breeding experiments of the usual genetical kind may demonstrate that certain characters are inherited through the cytoplasm and not through the nucleus and thus provide evidence for cytoplasmic hereditary determinants. A different type of evidence appears when it can be shown that a character can be transmitted from cell to cell by inoculation or other treatment with extracts which do not contain functional chromosomes; we may then conclude that we are confronted with a determinant, presumably derived from the cytoplasm, which can persist and impress some definite character onto the living cells into which it is introduced. The classical examples of such types of behaviour are the transmissible viruses. When from such evidence we deduce the existence of a plasmagene, it is presumably implied that the cytoplasmic determinant is a fairly complex body, probably of the order of magnitude of a virus particle or a gene. Considerable caution should be exercised in making such deductions. Many years ago, in the early years of the investigation of cancer-producing viruses, it was pointed out that, given a tissue which had an appropriate competence, a particular type of cellular differentiation could be transmitted through an indefinite series of inoculations by means of cell-free extracts whose operative factors, however, were quite simple molecules which acted as evocators (cf. Needham, 1936). One knows now that the effective molecules might be even simpler than was realized at that time. It would be quite possible to carry on an indefinite series of transformations of gastrula ectoderm into neural tissue by means of inoculations of cell-free extracts, provided only that these extracts were sufficiently acid. Moreover, one might easily obtain phenomena which

simulate a mutation of the virus. If the extracts came to contain free ammonia they would transform the gastrula ectoderm not into neural tissue but into derivatives of the axial mesoderm. More recently Lederberg (1952) has drawn attention to the same source of possible error. Thus to be justified in using such experiments to postulate the existence of a plasmagene, one needs evidence not only that the character can be transmitted by cell-free extracts but that the effective factor in the extracts is of the right order of complexity.

From the point of view of their possible importance in differentiation, plasmagenes may be considered under the following headings:

(1) *Exogenous*

Many viruses, such as those producing disease, are clearly not essential constituents of the animal or plant and are introduced into the cell from outside. There is considerable variation in the ease with which this introduction can take place. Some of the bodies which were originally thought of as true plasmagenes should perhaps be regarded as essentially exogenous factors for which infection is rather difficult. This probably applies to the kappa particles in *Paramecium* (Sonneborn 1951) and to the so-called genoid which confers CO_2 sensitivity on *Drosophila* (L'Heritier, 1948). One of the most important characteristics of plasmagenes of this kind is that they cannot be manufactured anew by the genes of the cell and thus if a cell is once free of them they will not appear again until introduced from outside. Apart from this fundamental fact, the relations between the exogenous plasmagene and the genotype of the cell may vary over a wide range. In some cases complete resistance to the exogenous particle may be controlled by a single nuclear gene. Thus in variety 4 of *Paramecium*, the kappa particle cannot persist in the cell unless that contains one or two doses of the nuclear gene K; the recessive form kk is completely resistant and cannot support the growth of the particles if they are introduced. In other cases, such as the *Drosophila* genoid and many viruses, there is as yet little evidence of nuclear control of susceptibility or resistance. On general grounds, however, it is probable that there is always some variation in this respect and this will probably be under the control of numerous nuclear genes, each of small effect.

Again, the physiological result of infection with the exogenous particle in some cases clearly depends on the nuclear constitution of the individual involved. The classical example is the virus-like particle in the King Edward race of potatoes, which has little effect in that stock but which, when transferred to other races of potato by grafting, produces the symptoms of a severe virus disease (Salaman and Le Pelley, 1930). In other cases such variation is less in evidence, but it seems likely that careful search would always reveal some degree of variability of this kind.

(2) *True plasmagenes*

One can pass by more or less insensible gradations from cases in which infection is easy and the infecting particle obviously not an essential component of the organism, to the other end of the range at which infection cannot be definitely demonstrated to occur, and the plasmagenes appear to be normal constituents of the organism. Particles coming at the latter end of the range may be considered as true plasmagenes. Most of the cases known are from the plant world (reviewed in Caspari,

1948). Perhaps the best investigated are the factors following clearly cytoplasmic inheritance in crosses between different races of the willow herb, *Epilobium*. Another example is provided by the cytoplasmic factors causing male sterility in crosses between races of a number of different species of plants (e.g. flax, maize, etc.). Evidence for such factors in higher animals is exceedingly rare. The case which perhaps seems most likely to fall into the category is that recently described by Laven (1953) in mosquitoes of the genus *Culex*. He shows that in crosses between certain species there is a factor, transmitted cytoplasmically through the eggs, which causes the sperm of the animals carrying it to be incompatible with the cytoplasm of the eggs of certain other races. This may be a true plasmagene. On the other hand, its sporadic occurrence in the races of *Culex* from certain localities, and the existence of rather similar factors in certain other nearly-related species of mosquito, raises the possibility that it may be rather an exogenous virus to which certain local races are adapted, while in others it produces the symptoms of damage to the sperm.

None of the entities in this category of true plasmagenes can yet be seen and there is no direct evidence as to the size of particle involved. The indirect evidence, chiefly from the type of physiological effect which they produce, is usually held to suggest that they are bodies of a gene-like order of complexity. It is not impossible, however, that in the future some of them may turn out to be simpler than has been previously thought.

(3) *Cytoplasmic particles with genetic continuity*

One should perhaps make a separate category for the comparatively large particles, visible with the microscope, which exist in the cytoplasm of some forms and are endowed with a genetic continuity; that is to say, in which each particle is normally, and perhaps always, derived from a similar previous particle. Examples are the kinetosomes in ciliates, and chloroplasts in green plants. They seem to differ from the true plasmagenes in the previous category not only in their larger size but also in the complexity of their relation with the nuclear genes. The true plasmagenes do not appear to be altered in their character by the nature of the nuclear genes with which they are associated, although they do, of course, enter into physiological relations with those genes during development. The character of the cytoplasmic particles, on the other hand, seems to be often, and perhaps always, under rather direct nuclear control. Very many genes are known in plants which control the formation of chlorophyl and the chloroplasts; and Weisz (1951) has described a most interesting set of interactions between the nucleus and the kinetosomes in the Protozoan *Stentor*, related to the differentiation of the regions of the body. There are in this form reciprocal interactions between the nucleus and the cytoplasmic particles. On the one hand the macronucleus controls the type of organelle (cilium or mouth part, etc.) which the kinetosome can form during regeneration: on the other, the kinetosomes in the posterior region of the body influence the nodes of the macronucleus which lie in that region, and deprive them of the capacity to cause the differentiation of anterior organelles.

(4) *Gene-initiated plasmagenes*

In contrast to the preceding categories there are a group of factors, which are also often considered to be plasmagenes, and which are characterized by the fact that

they can arise anew within cells from which they were originally absent. Their initiation seems in all cases to depend on the functioning of corresponding genes in the nucleus and is impossible if the effective gene is absent. Other conditions of an environmental kind are usually necessary to bring the gene into play and cause it to produce the cytoplasmic factor. Some of the best examples are the cytoplasmic determinants of antogenic specificity in *Paramecium* studied by Sonneborn and Beale (Beale, 1952). In these organisms, the environmental conditions control the state of the cytoplasm. When the cytoplasm is brought into a new state, this may initiate a process by which one particular gene, out of a group of possible ones, causes the appearance of a cytoplasmic factor, which in turn controls the formation of a corresponding antigen. Once they are formed, the cytoplasmic factors have some degree of autonomy, in that they can continue to function after the gene which initiated them has been removed by crossing. But this autonomy is very limited; they persist for only five generations after the gene is removed. If, on the other hand, the environment is changed to one which will eventually bring another gene into operation, the formation of the original antigen may continue for a considerably longer time; but in this situation the gene corresponding to the cytoplasmic antigen-producing factor is still present in the nucleus, and what persists is the complex of gene and cytoplasmic factor rather than the latter alone.

Another example is the factor studied by Billingham and Medawar (1948) which produces melanin pigmentation in the skin of the guinea-pig. This factor is detected by the ability of black pigment-producing cells to infect neighbouring unpigmented cells with the ability to deposit melanin. The possibility of a serial evocation needs to be considered here, but it appears that the factor which is transmitted during infection carries with it an immunological specificity which is too precise to be attributed to a simple evocator molecule. The infective agent is gene-dependent, in the sense that it only appears in the skin of animals whose genotype contains the factors for the appearance of melanin pigment. Again, in individuals of this genotype it appears only in the cells of the pigment-forming system, that is to say the derivatives of the neural crest. The activity of the genes responsible for its production must therefore be dependent on the internal environmental factors which control the differentiation of this tissue.

Finally we may mention the examples of adaptive enzyme formation in yeasts and bacteria, etc., in which the conjunction of a particular substrate in the medium and an appropriate gene in the nucleus seems to be necessary for the production of the enzyme, the immediately effective agent being, according to some authors (e.g. Spiegelman, 1951) a plasmagene-like factor in the cytoplasm. The factors concerned with the formation of respiratory enzymes in yeast studied by Ephrussi (1953) probably are somewhat similar in character.

It is questionable whether it is really appropriate to employ the word 'plasmagenes' for these gene-initiated factors. The character they share with the true plasmagenes is that of a certain ability to multiply through a number of cell generations after the gene which initially endowed them with their specific characteristics has been removed. It is not clear, however, that any case is known in which a gene-initiated cytoplasmic factor possesses complete autonomy in its powers of reproduction. Certainly the *Paramecium* antigen determinants can only persist for a very

limited period after the removal of the gene. The situation of the factors studied by Billingham and Medawar is obscure, since in the piebald guinea-pigs they studied the originally colourless cells into which the factor passes probably possess the same genotypic constitution as the coloured cells out of which it comes, the difference between the cells being one which arises during differentiation rather than one of a genetic nature. Beale (1954), who had studied these phenomena as closely as anyone, has recently expressed a lack of satisfaction with the term 'plasmagene' for such factors. Haldane (1954) is apparently of a similar opinion and has suggested calling them 'mnemons'. For convenience in the present discussion, however, I shall continue to refer to them as 'gene-initiated plasmagenes'.

It will be noticed that the overwhelming bulk of the evidence for the existence of plasmagenes comes from studies on micro-organisms. It might be, however, that this is caused not by their rarity in other forms but by factors which make their detection particularly difficult. It is clear, for instance, that if plasmagenes were to play an important part in the differentiation of multi-cellular organisms, they could not in general be capable of easy infective transmission from one cell to another, since that would lead to an intermingling of different organs or types of tissue which should remain separate. Thus we cannot expect to find many cases similar to that of Billingham and Medawar, even if factors of an essentially similar nature were widespread. It is necessary, therefore, to approach the matter to some extent from an *a priori* point of view to try to determine how far plasmagene-like factors could fit in to the mechanisms of differentiation in so far as we understand them at present.

It is clear that the exogenous factors mentioned under group (1) above do not come into the question. In the examples of the true plasmagenes mentioned in group (2), the cytoplasmic determinant is a part of the general genetic constitution of the organism and no more directly related to the differentiation of its various parts than are the nuclear genes. It is, however, possible to imagine that the cytoplasm of the egg of a given species might contain a number of different true plasmagenes localized in various regions. Each region of the egg would then contain characteristic cytoplasmic factors endowed with genetic continuity which might determine the nature of the organs which develop out of it. Such localized plasmagenes would, in fact, be the same thing as used to be referred to at the beginning of this century as 'organ-forming substances'. Now there is no doubt that in many eggs different regions of the cytoplasm have different properties. The regions concerned are nowadays referred to as 'oöplasms', and opinion has rather moved against attributing their properties to the presence of substances which are autonomous over against the nucleus.

The arguments which have swayed opinion against the old idea of organ-forming substances are numerous. One is that the evidence suggests that the oöplasms are only effective when they are able to interact with the nuclei; for instance, Seidel (1929) has shown clearly that the cytoplasmic activation centre in the posterior of an insect egg only becomes active when nuclei reach it. Spemann (1938) produced similar evidence in connexion with the grey crescent oöplasm of the amphibian egg. Again, as we saw in the case of the eye, discussed at the beginning of this paper, differentiation from the egg to the final form takes place in a series of steps. It does not look as though we are dealing merely with the sorting out of a number of factors which

from the beginning preserve their character unchanged, but rather as if we are dealing with a series of reactions during which the state of the system continuously changes until the final condition is gradually built up. We are already faced with the difficulty of accounting for this progressive series of changes in a system one of whose major components consists of genes, which we believe to retain their identity throughout. The difficulty is only made the greater if we have to suppose that the major factors in the cytoplasm also retain their identity.

As a third argument, one may point to the fact that the localization of different organs within the developing body may often be altered by factors which operate after the segregation of plasmagenes in the egg cytoplasm must have been completed. For instance, one might be tempted to attribute the localization of the organs in a developing *Drosophila* to the segregation of organ-forming substances or plasmagenes in the eggs, which are known to belong to the mosaic type; yet an environmental treatment applied many hours after fertilization can cause an extra mesothorax to develop instead of the normal metathorax (Gloor, 1947). Even as late as the third larval instar environmental treatments (temperature shocks) applied to flies homozygous for aristapedia can change the proportion of the antennal bud which develops into a leg-like organ or into an arista (Vogt, 1946). The mere fact that a gene, like aristapedia, can cause a mass of tissue which should normally develop into an antenna to develop into a leg instead, shows that, even if we try to attribute the major processes of differentiation to plasmagene-like bodies, these bodies cannot be autonomous in their properties but must be highly susceptible to modifications caused by interaction with genes.

It appears, therefore, that the postulation of true plasmagenes as organ-forming substances in the cytoplasm of the egg does not materially simplify the theoretical task of accounting for the phenomena of differentiation. That does not necessarily mean, of course, that such bodies do not or cannot exist; we should have to take account of them if there was unequivocal evidence for the existence in the eggs of multi-cellular animals of cytoplasmic factors which had genetic continuity independently of the nucleus. As yet I know of no compelling evidence to this effect. Indeed, attempts to assess the autonomy of cytoplasmic factors in the egg over against the nucleus have been few and far between. The brilliant studies of Baltzer and his pupils (cf. Baltzer, 1950) with hybrid merogons in amphibia are perhaps the most promising. So far as I know, the facts there can all be accounted for in terms of a mere persistence of cytoplasmic character, without the need to postulate that the cytoplasmic factors can reproduce while retaining their specific nature. One may conclude, I think, that there is hardly any evidence that plasmagenes with complete autonomous genetic continuity exist in metozoan eggs, and that it seems most improbable that the major phenomena of differentiation can be attributed to them.

The same conclusion applies even more forcibly to the plasmagenes of category (3), namely microscopically visible cytoplasmic particles with genetic continuity. These certainly occur in certain special cases, as for instance in ciliates, but in general the histological evidence makes it clear that differentiation does not consist to any major extent of the mere sorting out of the already existing particles in the egg cytoplasm. Such particles probably play an important part in development, but not by the mere retention of their original characteristics.

The situation is rather different when we turn to the fourth category, that of gene-initiated plasmagenes. If these were to play an important part in development we should have to imagine that the various oöplasms of the egg differentially excite the nuclei which enter them; that the particular genes which are activated in a given region then cause the appearance of cytoplasmic factors, and that these factors, when they have appeared, show a certain degree of autonomy, being able to reproduce for a short time with repetition of their character even if the nucleus is later removed. If one supposes that, once they have been formed, the autonomy of the plasmagenes is complete, this suggestion would come up against the same difficulties as confronted the hypothesis of organ-forming substances in accounting for the sequential character of differentiation, and phenomena such as the metaplasia of retinal cells into lens in Wolffian regeneration. We have seen, however, that in the best-studied examples of gene-initiated plasmagenes the autonomy is by no means complete. If one waters it down sufficiently, the difficulties which have just been mentioned could be overcome. The hypothesis would then amount to the suggestion that during differentiation the genes cause the appearance in the cytoplasm of bodies with a certain limited amount of autonomy. There seems nothing impossible, or even very difficult, in such a suggestion. Brachet (1944, 1952) has urged it with considerable persuasiveness. He points to a type of cytoplasmic particle, the ultra-centrifugable ribose-nucleic acid-containing microsomes, which he supposes to be the plasmagene-like factors in question. The problem that still remains at issue is how far these particles, once their character has been determined, become independent of the nucleus. Only the transplantation either of the nuclei or of the particles from one type of differentiating cell to another can settle the matter conclusively. There would, in my opinion, be nothing surprising if experiment eventually showed that, in cells which are more or less completely determined and are in process of producing their final cytoplasmic constituents, the cytoplasm is able to carry on in synthesizing these more or less independently of the nucleus. However, such a fact does not yet seem to have been demonstrated. Whether, if it were, we should be justified in speaking of the effectiveness of plasmagenes in differentiation would be largely a matter of definition; it would depend on whether we are satisfied that such gene-initiated cytoplasmic factors of limited autonomy are comparable to plasmagenes of the more classical kind.

Turning now from plasmagenes, let us consider some of the more general characteristics of the physiological processes in a developing cell. The basic fact which we have to try to understand is that different cells in the body, although presumably all containing the same genes, yet differentiate into quite different tissues. The fundamental mechanism must be one by which the different cytoplasms, or oöplasms, which characterize the various regions of the egg, act differentially on the nuclei so as to encourage the activity of certain genes in one region, of other genes in other places. Such specific activation of particular genes at certain times and places can actually be observed visually in favourable cases, for instance, in the important work of Pavan (1954), Mechelke (1953) and Beerman (1952) on the polytene chromosomes in various tissues of chironomids. The fact of differential activation of genes is, then, scarcely in doubt. But there has been as yet little discussion of how we may envisage such a process in chemical terms. There are innumerable different types of kinetic system which might be supposed to be in operation, but it is worth some further

exploration of the simpler varieties of these to give a rather more definite picture of the kind of system with which we are confronted.

Let us consider, at first, two substances P and Q, which are being formed out of the raw materials A, B and C, for the supplies of which they compete. To give the simplest possible picture of such a competition, suppose that P is formed from A and B, while Q is formed from B and C. Again for the sake of simplicity, let the reaction constants be the same for the two syntheses, as shown in Figure 1; and let us

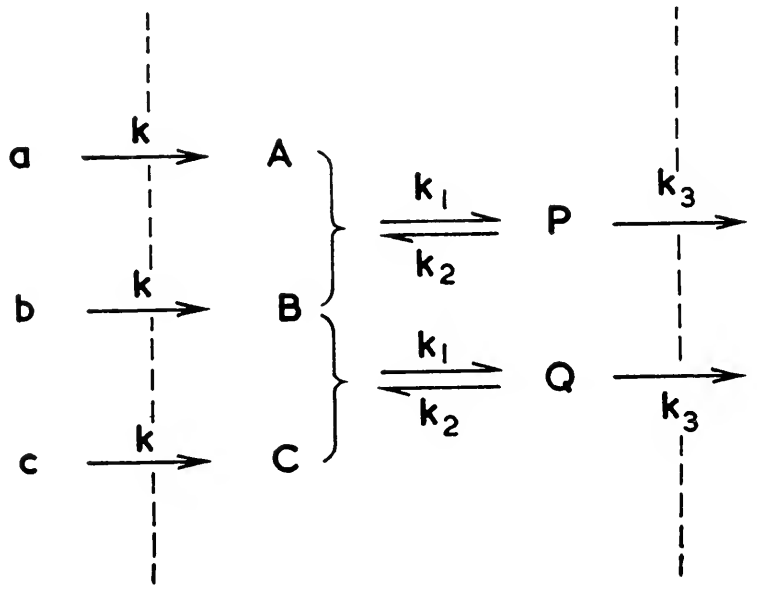

Figure 1.

suppose that A, B and C diffuse into the system at rates proportional to the difference in their concentration inside (A, B, C) and outside (a, b, c), while P and Q are removed at rates k_3. Then for the rates of change of the various components of the system we shall have a set of equations:

$$\frac{dA}{dt} = k(a - A) - k_1AB + k_2P$$

$$\frac{dB}{dt} = k(b - B) - k_1AB + k_2P - k_1BC + k_2Q$$

$$\frac{dC}{dt} = k(c - C) - k_1BC + k_2Q$$

$$\frac{dP}{dt} = k_1AB - k_2P - k_3P$$

$$\frac{dQ}{dt} = k_1BC - k_2Q - k_3Q$$

The system will change progressively. The course of the change is complicated to describe in detail, but we can discover something about the general characteristics of the system if we consider only the final steady state, when no further change is occurring, and the right-hand side of each equation is equal to 0. Under these conditions it is easy to show that the ratio P/Q will be equal to a/c. That is to say, if in one region of the egg, the available supplies of A are increased in comparison with those in some other region, then the steady-state concentration of P in the first region will be increased in exact proportion. That is, of course, not very surprising. And it hardly seems to provide much enlightenment as to the mechanisms of differentiation. What we seem to meet in embryology are situations in which small initial differences lead to large divergences in later development. To account for this, we need something more complicated than the very simple system we have just discussed.

As a first step towards a more adequate picture, let us suppose that the coupling of A and B to form P, and of B and C to form Q, are autocatalytic processes, i.e. are speeded up by the presence of already-formed P and Q. This is a simple form of a 'feed-back' mechanism. Our equations will now be

$$\frac{dA}{dt} = k(a - A) - k_1PAB + k_2P^2$$

$$\frac{dB}{dt} = k(b - B) - k_1PAB + k_2P^2 - k_1QBC + k_2Q^2$$

$$\frac{dC}{dt} = k(c - C) - k_1QBC + k_2Q^2$$

$$\frac{dP}{dt} = k_1PAB - k_2P^2 - k_3P$$

$$\frac{dQ}{dt} = k_1QBC - k_2Q^2 - k_3Q$$

At the steady state, we find a relation between P and Q of the form

$$(kk_2c + k_3^2)P = (kk_2a + k_3^2)Q + kk_3(a - c) \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

(Note that although the dimensions in this look a bit odd at first sight, k and k_3 are simple diffusion constants, while k_1 and k_2 are rate constants of third- and second-order reactions.)

Now if k_3 is small compared with k (i.e. diffusion out of the system is slower than diffusion in), then we can neglect its higher powers, and we find

$$P = \frac{a}{c}Q + \frac{k_3}{k_2}\frac{a - c}{c} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

Thus if initially in a certain region the supply of A is increased relative to that of C, we find that P will be increased relative to Q by something more than a proportionate amount, the excess being expressed by the last term on the right. And if the rate of removal of P (that is k_3), is greater than the rate at which P breaks down again into A and B, (that is k_2), this excess can be considerable. We can also see from expression (2) that the exaggeration will be the more important the smaller the

absolute values of P and Q; and these will also be reduced if k_3 is fairly large, so that P and Q are rapidly removed.

Without going into further details, we can see that if two autocatalytic processes compete for raw materials, we may under some conditions find that an initial change in the supply of the materials produces an exaggerated effect on the steady-state concentrations of the synthesized products, and thus on the rates at which these products can be made available outside the system.

If we suppose that a, b and c are the raw materials out of which two genes manufacture their immediate products P and Q, we have now developed a picture by means of which we can see how change in the concentrations of these raw materials leads to exaggerated differences in the rate at which P and Q are passed out of the nucleus into the cytoplasm. I have previously suggested this model, without going into such detail concerning it (Waddington, 1948).

It is, however, by no means the only model which might be appropriate. As Delbrück (1949) has suggested, there might be direct interactions between the two synthetic processes. These are perhaps most simply formulated by supposing that P is destroyed at some rate proportional to the concentration of Q (and vice versa). The equations for $\frac{dP}{dt}$ and $\frac{dQ}{dt}$ will then contain terms in PQ. If we regard the system as closed, rather than open as was the system discussed above, and if the supplies of raw materials are taken as constant, the equations which result are of the same type as those which arise in the study of the growth of two populations of animals which compete with one another for a limited food supply. Lotka (1934) had discussed the relatively simple situation of two populations (or substances) for which the equations take the form

$$\frac{dP}{dt} = m_p P - k_p P^2 - k_{pq} PQ$$

$$\frac{dQ}{dt} = m_q Q - k_q Q^2 - k_{qp} PQ$$

He shows that according as $m_p k_q$ is greater or less than $m_q k_{pq}$, and $m_p k_{qp}$ greater or less than $m_q k_p$, so the final state of the system is either wholly P, or wholly Q, or a certain fixed ratio between them, or finally the system is one which will finish up either entirely P or entirely Q according to the initial concentrations of these substances.

Again, Kostitzin (1937) discusses a somewhat similar set of equations which he takes to represent two species competing for a food supply which consists of another species which multiplies in the normal way, but which could equally well represent two autocatalytic substances which interfere with one another and also compete for a raw material which is supplied at a more or less exponential rate. In this case also he shows that, under some conditions, the system will be such that the initial conditions will determine whether it goes wholly in the direction of one of the competing elements or in that of the other.

Kostitzin has also discussed shortly the more general case in which there are many competing and interacting substances (or populations), so that we have a large series.

of simultaneous differential equations, each containing terms of the second order, such as p^2 or pq etc. He shows that such a system may be expected to exhibit a number of alternative steady states, some at least of which are likely to be stable, and that the particular one which the system actually attains will in many cases depend on the initial conditions.

This sort of investigation seems to me to provide the bare skeleton of the theoretical outlook which we require to understand the mechanisms of differentiation. The field would, I think, repay much more study than it has yet received. In particular, one would like to see a further discussion in terms of open systems rather than closed ones. I hope that some competent mathematician, at home in the field of chemical kinetics, will interest himself in it.

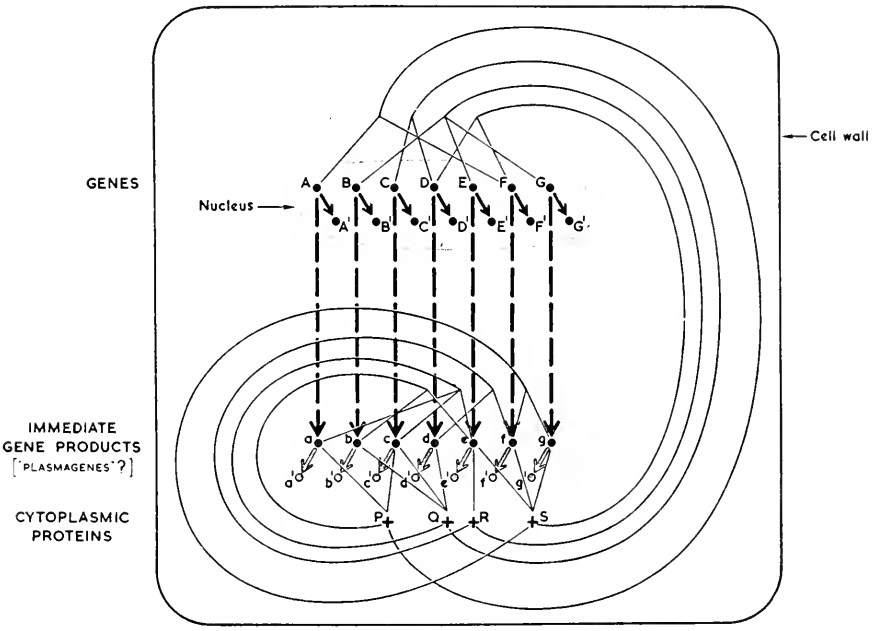

Figure 2. The double cycle of processes in a differentiating cell.

I should like to pass on to the last point I want to mention about the physiology of the developing cell. The genes both draw the raw materials they use to make their gene-products from the cytoplasm, and pass the products themselves back into the cytoplasm. Similarly the raw materials for the synthetic processes going on in the cytoplasm (controlled by the immediate gene products, either with or without the intervention of plasmagene-like factors) are taken from the cytoplasm while the synthesized products are returned to it. We must therefore expect to have to deal with cyclical processes. In fact, there must be two such cycles, one by which the cytoplasm controls the activity of the genes while the latter change the character of the cytoplasm; and a second similar cycle involving the immediate gene products rather than the genes themselves. The system of the developing cell can therefore hardly be more simple than that represented by the diagram Figure 2. If some of the

immediate gene products, or elements close to them in the reaction sequence, act as gene-initiated plasmagenes, and have the capacity for identical self-duplication, we should have to add to the diagram some further thick arrows similar to those which symbolize the identical multiplication of the genes themselves. But until the empirical facts force us to recognize the existence of such bodies, there seems no theoretical need to postulate them.

It is easy to see that in such cyclical systems, the switch mechanisms we have discussed, which serve to exaggerate the effects of small differences in initial conditions, may be even more powerful. This would be so if the products of gene activity increased the supplies or the suitability of the raw materials which that gene required.

It follows from all this discussion that normal chemical kinetic theory provides several different models for systems of interacting synthetic processes which will change progressively towards one or other of a number of alternative steady states, the decision between the various potential directions being made by the initial conditions. These are just the characteristics which experimental analysis leads us to attribute to developing cells. It is in terms of models of this kind that we must envisage the fundamental processes of developmental physiology. The occurrence of gene-initiated plasmagenes with a limited autonomy is a possibility, but whether they are actually of general occurrence must be left for future experiments to decide. But in themselves they do not provide an adequate explanation either of the progressive character of development, or of the occurrence of alternative end-states. They should be regarded as possible elements in cyclical reaction systems of the kind we have discussed rather than as providing an alternative model for the understanding of development.

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The time-graded regeneration field in planarians and some of its cyto-physiological implications

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THE TIME-GRADED FIELD

PLANARIAN regeneration has received much attention because these animals, especially the fresh-water triclads, seem to be 'immortal under the edge of the knife', to use an impressive phrase of Dalyell (1814).

Prominent biologists have sharpened their experimental arts and their scientific wit in their efforts to solve some of the deep-rooted riddles immanent in the spectacular powers of regeneration exhibited by these inconspicuous animals.

T. H. Morgan and C. M. Child have been among the foremost in this research work, and some far-reaching hypotheses regarding morphogenesis in general have emanated from this work, such as the well-known gradient hypothesis of Child.

The tide of research on planarian regeneration has flowed and ebbed, as in most other scientific disciplines. Now we are in a period of rising water, thanks in part to the brilliant work carried out in Wolff's laboratory in Strasbourg. The two outstanding results are (1) Wolff's and Dubois' (1947) and Dubois' (1949) demonstration that neoblasts, totipotent embryonic cells, form the regeneration blastema and hence are responsible for the rebuilding of the missing parts, and (2) Wolff's and Lender's (1950, 1951) finding that eye-formation in *Polycelis* depends on the presence of head ganglia. Other lines of approach have been followed in my laboratory for several years; one of these I should like to outline before you to-day.

We know from the earliest experiments, notably those of Morgan (1902), that in some planarian species every part of the body has the power to regenerate a whole animal; thus every part of these species is able to regenerate a head from an anterior wound. We may therefore ask: why do not many heads regenerate from the anterior wound of a transected animal?

Figures 1 and 2 show some instances of heads formed at rather unexpected places; in the second instance regeneration occurs in spite of the fact that the old head was not cut away—which, incidentally, shows that no inhibitory force for head-regeneration can travel through adult tissue. The heads at the 'arms' in Figure 1 and the head in the window in Figure 2 are regenerated at a much lower speed than a median head from an anterior transverse cut. This suggested that *time* was involved in regeneration of only one head from a surface where the potentiality of plural head-regeneration was present.

Accordingly, several sets of experiments were carried out with the purpose of finding out the rate of head-regeneration from various parts of the body. The experiments were made by cutting the animal in pieces according to a certain pattern and noting the time necessary for the pieces to regenerate heads. (Brøndsted, 1946; A. and H. V. Brøndsted, 1952). In this way it was found that there existed a static time-graded regeneration field, different for each planarian species so far investigated. Figure 3 shows the extent and intensity of the field in *Bdellocephala*. The field is static in the unwounded animal, and of course only displays itself after a cut has been made.

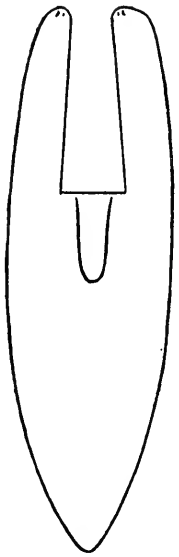

Figure 1. Bdellocephala punctata *after decapitation. The median part has been removed; eyes are regenerated at both 'arms'.* (*Brøndsted*, 1946.)

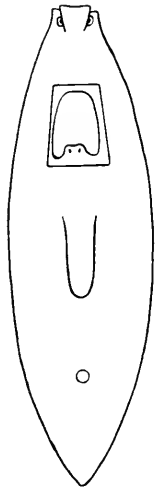

Figure 2. Bdellocephala punctata. *A quadrangular piece has been removed; a head is regenerated at the anteriorly directed wound surface at the posterior side of the 'window'.* (*Brøndsted*, 1946.)

The significance of such a dynamic structure is apparent. When the rate of regeneration varies in the way shown, every cut will expose a surface in which some place has the highest regeneration rate; this place is called the *high-point*. From here regeneration of the head starts, but in doing so it must at the same time inhibit neighbouring parts from exercising their ability to make heads themselves. Thus the time-graded regeneration field, together with an inhibitory action exercised from the high-point towards neighbouring parts, ensures that only one head is formed from the anterior-facing surface of a wound, and so leads to harmonious regeneration. In order to test this hypothesis some experiments were carried out, of which a full account will be published elsewhere. These experiments were designed to demonstrate the inhibitory influence which must pass from the high-point to the

sides before these have had time to start irreversibly along the head-determining line.

A transverse section is cut out of a great number of *Dendrocoelum*; the cuts must be made at the same level in the time-graded field in all specimens. The segments are divided into five equal lots. In the first lot a lateral third is isolated by a longitudinal

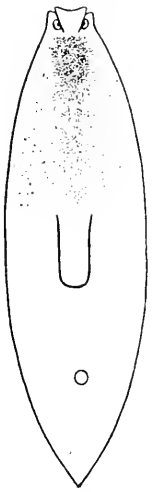

Figure 3. Bdellocephala punctata. *The time-graded regeneration field; darkest shading represents highest regeneration rate.*

cut, as shown in Figure 4, 24 hours after the segment has been cut out of the animal. The second lot is handled in the same way after 48 hours, and so on until the fifth lot has had its lateral part cut away after 120 hours.

We know that in *Dendrocoelum* a lateral third, when isolated, takes 7 days to regenerate a head. If an inhibitory force emanates from the median high-point, the lateral

Figure 4. *Transverse anterior segments of* Dendrocoelum. *The signs are those used in the curves of Figure* 5. *Further explanation in text.*

piece, when isolated after a certain time, should now require more time to regenerate a head.

Figure 5 shows the results of some experiments. It is clear that an inhibition actually sets in at some time between 72 and 96 hours after the segments were cut out. Lateral pieces isolated after this time require more time for head regeneration than pieces which were isolated during the first 72 hours.

The distance which the inhibitory influence has to traverse is roughly about 500 microns; if the size of a neoblast is about 10 microns, then 50 cells transmit the

influence in, say, 80 hours, giving an average of 100 minutes per cell. This may be of future interest.

There is, however, a further point which must be considered concerning the isolating experiment. When the median high-point has regenerated a head on the anterior-facing surface of a transverse cut, the time-graded field will be restored in accordance

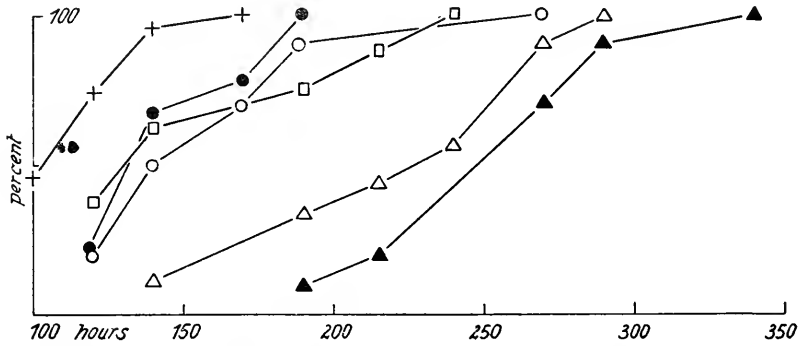

Figure 5. *Eye-formation in lateral pieces of transverse segments of* Dendrocoelum. + *median part;* ● *lateral parts isolated after* 24 *hours;* □ *after* 48 *hours;* ○ *after* 72 *hours;* △ *after* 96 *hours;* ▲ *after* 120 *hours.*

with the shortened axis of the animal; therefore, when regeneration has proceeded for a certain time, the lateral parts of the segment just behind the new head will have reached a level in the time-graded field characteristic of the intact animal. Moreover, the regeneration processes have made the animal normal, the new head is no longer a blastema, the differentiation processes are nearly accomplished, and we know that inhibition from a differentiated head does not occur. It follows from these considerations that if the above-mentioned experiment were prolonged after 120 hours we

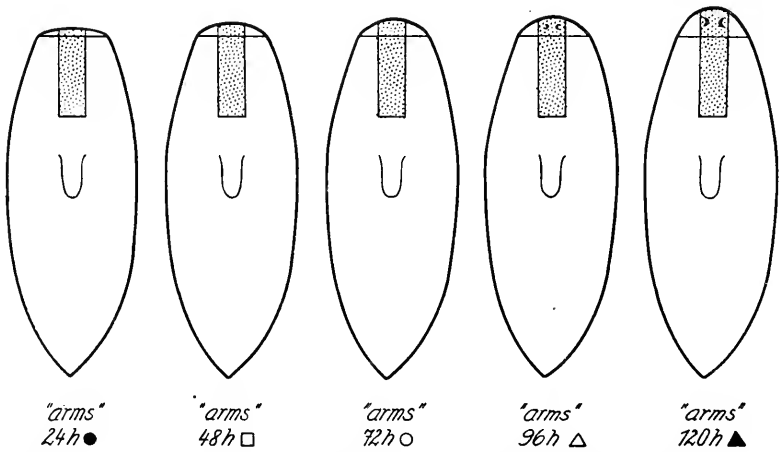

Figure 6. Bdellocephala punctata. *The median stippled parts of five lots of animals have been removed after the times indicated. Note that lot* 4 *at the time of removal already has regenerated eyes.*

should expect a shortening of the time required for head-regeneration in lateral pieces isolated after 144, 168 hours, etc.

This experiment was carried out on another species, *Bdellocephala punctata*, and in a slightly different way. Figure 6 shows the procedure which was adopted in order to see whether a connexion with the main body would make any difference in

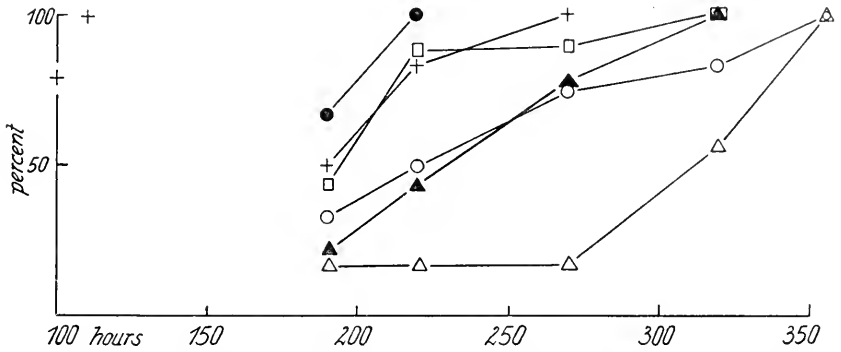

Figure 7. Eye-formation at 'arms' in Bdellocephala. ● *'arms' isolated immediately after decapitation;* + *arms isolated after 24 hours;* □ *after 48 hours;* ○ *after 72 hours;* △ *after 96 hours;* ▲ *after 120 hours.*

the inhibitory effect found in *Dendrocoelum*. Figure 7 shows that it did not. A difference in the time-relations appeared, however, owing to the higher rate of head-regeneration from the median high-point in *Bdellocephala*. Already after 96 hours eyes

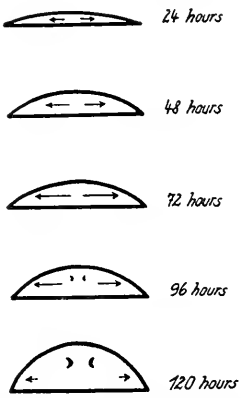

Figure 8. Schematic representation of the rising and declining inhibitory force during regeneration of a median head.

are discernible in this species, and the head is well established after 120 hours. Thus the inhibitory force from the median high-point is declining after between 96 and 120 hours; so that the lateral parts, or 'arms', are only under a slight inhibitory influence after 120 hours, and accordingly they regenerate heads somewhat faster than the 'arms' isolated after 96 hours. This is shown schematically in Figure 8.

We know nothing definite as yet as to the nature of the time-graded regeneration field in planarians. However, a series of transplantation experiments has revealed that it must have a rather firm structural base; humoral conditioning is, as far as I can see, out of the question. Some of these experiments will now be mentioned. The transplantation technique has already been described (Brøndsted, 1939).

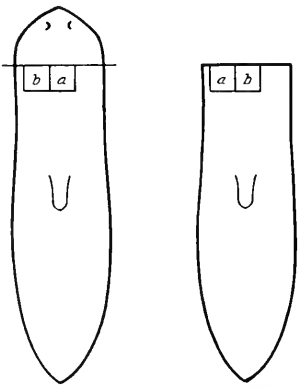

Figure 9. Planaria lugubris. *After decapitation a median part is exchanged with a lateral part.*

If in *Planaria lugubris* the median part of the field is exchanged after decapitation with a lateral part (Figure 9), these two parts will regenerate heads at the normal rate characteristic of the level of the cut, irrespective of the new situation; the median will not be slowed down, nor will the lateral be accelerated. An example is shown in Figure 10.

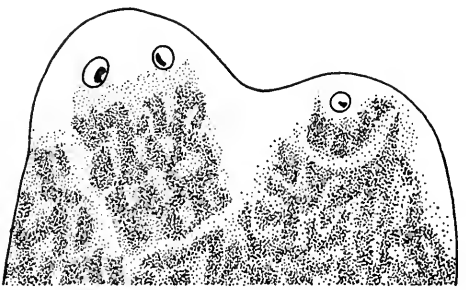

Figure 10. Planaria lugubris. *The interchange of* a *and* b *as seen in Figure* 9 *does not produce any effect on the rate of regeneration inherent in a median and a lateral piece.*

If anterior transverse segments are interchanged with posterior ones in such a way that the posterior pieces take up the position of the anterior ones, head-regeneration from the anterior wound surface will proceed at the rate characteristic of the level at which that wound was made (Figures 11–12).

In *Bdellocephala* no head-regeneration occurs at wound surfaces in the hindmost three-fifths of the body. If a transverse segment from the forepart is bisected along the median line, a posterior segment likewise, and the anterior half transplanted to a

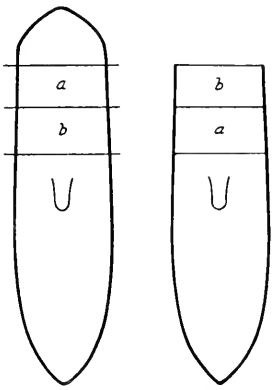

Figure 11. Planaria lugubris. *After decapitation* a *was interchanged with* b*; head-regeneration followed at the rate inherent for* b*-level.*

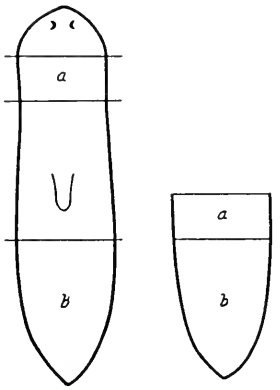

Figure 12. Planaria lugubris. *The* a *segment was transplanted to* b*; head-regeneration from* a *followed at the rate inherent for the* a*-level.*

posterior half, the two will build a common blastema, but only the anterior half will regenerate a head, and this is so both with normal and reversed polarity (Figures 13–14).

Isolated halves will start head-regeneration by making their 'own' half head first; a left half will regenerate a left eye, only later also a right eye. That is to say that the

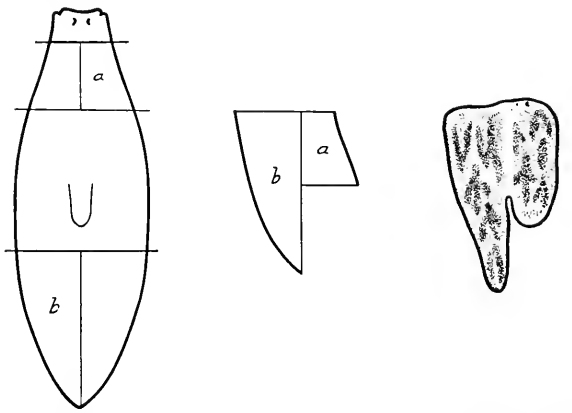

Figure 13. Bdellocephala. *The segment* a *was transplanted to* b *with the same polarity; only* a *regenerates head.*

time-graded regeneration field is, in reality, two fields, a left and a right. Investigations into the relations of the two symmetrical fields may therefore throw some light on the problem of bilaterality.

The time-graded regeneration field in *Planaria lugubris* extends all over the body.

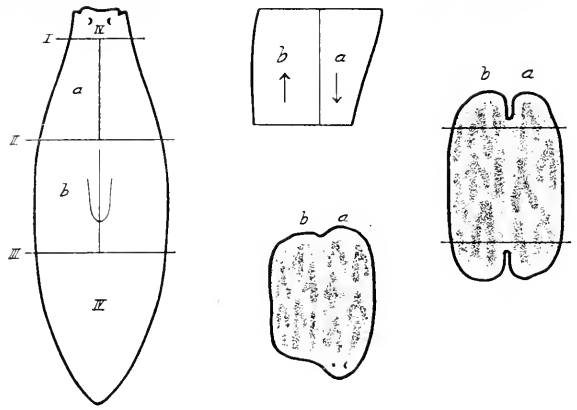

Figure 14. Bdellocephala. *The segment* a *was transplanted to* b *with reversed polarity; after severe wound closing two transverse cuts were made through the chimera;* a *regenerated a head with rate and polarity characteristic of the portion.*

I therefore asked some questions of this planarian, using a rather crude mode of interrogation. I made a cut in the median line of the animals and—after removing the protruding pharynxes—immediately reunited the two halves in such a way that they were shifted along the longitudinal axis. After a few days the two halves had healed together, and the animal was then decapitated by a transverse cut. The

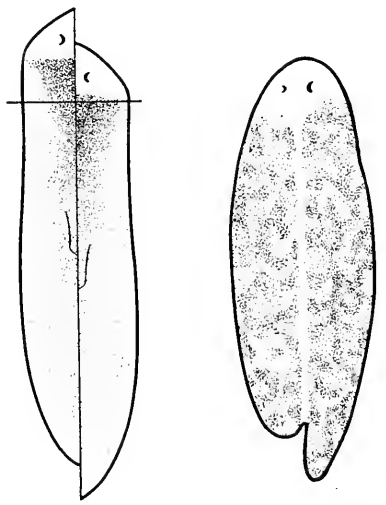

Figure 15. Planaria lugubris. *Two halves were reunited with a slight shift. A transverse cut was made through the fields as indicated in the left figure. The right figure shows the resulting normal animal; only the right eye is slightly bigger.*

purpose of the experiment is of course to cut across the time-graded field at different levels in the two halves.

As a result of this treatment, a blastema was formed all over the anterior transverse wound. Three distinct types of regeneration followed.

If the shift was slight a normal symmetrical head would regenerate, although the eye was a little bigger in the part of the blastema belonging to the half where the cut had hit a higher level; this indicates of course a faster regeneration rate (Figure 15).

If the shift was severe, head-regeneration only occurred in the half in which the cut had hit a high level of the field; the head in the other half was inhibited (Figures 16 and 17).

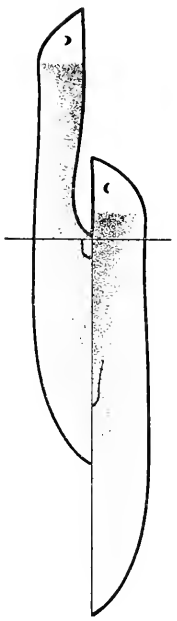

Figure 16. Planaria lugubris. *Two halves were reunited with a severe shift; the transverse line indicates the cut made after healing.*

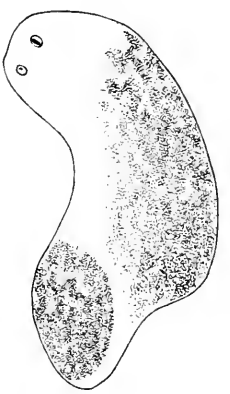

Figure 17. Planaria lugubris. *Head-regeneration only occurred in the right half after the operation indicated in Figure* 16.

In a few instances, namely when the shift was intermediate between slight and severe, each half of the common blastema regenerated a complete head (Figures 18 and 19); this was rather puzzling, but it is in fact as would be expected.

Let us consider what we have learnt about inhibiting forces emanating from the high-point, and we shall see that the results of these experiments agree with the inhibition hypothesis.

If the shift has only been slight, the inhibiting force from the right half of the blastema will not have time enough to inhibit eye-formation in the left half before this one has carried its differentiation so far that it has not only made its own left eye but also has set up inhibition towards the right half, in which, accordingly, regeneration of a left eye is inhibited.

If the shift, however, has been severe, the half blastema with the high regeneration rate will have time enough to regenerate not only its own eye but also the symmetrical one, because no inhibiting force has been set up yet in the other half; this other half is under so strong an inhibitory force that, on account of its very slow regeneration rate, it has no chance whatever to differentiate eyes.

Between these two conditions there must be one in which the relations between the two halves of the blastema are such that both of them may get time enough to make

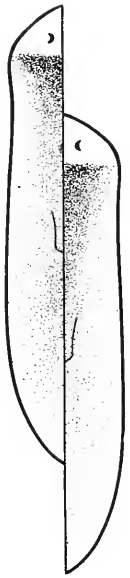

Figure 18. Planaria lugubris. *Two halves reunited with moderate shift. A transverse cut was made in the foremost part of the right piece hitting the field at a high level, but in the left half hitting the field at a somewhat lower level.*

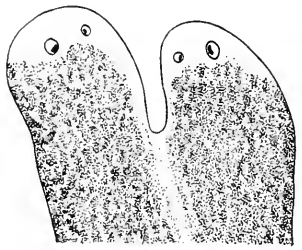

Figure 19. Planaria lugubris. *The two-headed chimera resulting from the operation indicated in Figure* 18.

not only their own eye but also a symmetrical one *before* the inhibiting influence from the other half has reached them.

If an amphibian egg in the two-cell stage is transected into two separate cells, provided the first cleavage furrow has taken place near the median plane, each of them will regenerate the missing part, and twin embryos are formed. It is just the same when a planarian is split longitudinally: two worms will come forth.

It is also true that if one of two amphibian blastomeres is killed by cautery, but otherwise left in its old position as a symmetrical part of the cleaved egg, the living blastomere will continue its development into a half embryo. That is to say, the killed blastomere contains inhibitory forces exercising their influence for a long period of time.

These are the chief facts so far discovered concerning the time-graded regeneration field in planarians.

As to the nature of the field I have only a few suggestions. It may be that the structural foundation is the nervous system; I do not think so, however, because there seems to be no correlation between the pattern of the nervous system and that of the field. More probable is the notion that the quantity of neoblasts determines the rate of regeneration. Some observations support this view, others do not. The question can of course only be answered by counting the neoblasts; this counting is being done in my laboratory, but I can as yet give no figures. But even if the quantity of neoblasts were to determine the rate of regeneration, we are still ignorant of the factors which determine a strict species-specific distribution of neoblasts, which otherwise can easily wander freely about in the planarian body. Here it might be suggested that some diffusable substance from the head ganglia attracts the neoblasts; such a mechanism might account for a caudally tapering concentration of the neoblast population.

The facts so far revealed about the time-graded regeneration field have led me to some considerations concerning three major problems in morphogenesis: *polarity*, *inhibition*, and *gene-action*.

POLARITY

We perceive polarity in single cells and in whole multicellular organisms. This has led some authors to postulate polarity in every single cell in the organism, but I think without justification. In the intact planarian body the neoblasts cannot be polarized. Neoblasts from a certain region may migrate to an anterior wound and regenerate a head; or they may migrate to a posterior wound and regenerate a tail-tip; they may also migrate to a lateral wound and regenerate missing side parts of the body. *The neoblasts are totipotent.*

The neoblasts are compelled to start regeneration in a certain main direction; the directive force must come from the remaining part of the body. For instance in *Planaria lugubris* a blastema formed at an anterior-facing wound surface will always start by making a head, irrespective of the level along the main axis of the body. In the same way, a blastema at a caudal wound surface will always start by making a tail-tip, also irrespective of level. Here polarity displays itself. Exceptions will be dealt with later.

What is the nature of the mechanism? We know that the neoblasts forming the blastema are totipotent. We know that in the intact body they are under control and are inhibited from displaying their potentiality. But in an anterior blastema, for instance, the neoblasts are freed from inhibition by the forepart of the body, which was removed by the cut, but they are *not* freed from inhibition by the remaining hindpart of the body. The same holds true—*mutatis mutandis*—for a posterior blastema.

The blastema is to be compared with the anterior or posterior half of a blastula not yet determined. Hence the two kinds of blastemata will act accordingly, that is, they will start morphogenesis at opposite ends of the main axis, comparable with the most animal and most vegetal part of the egg. I cannot help thinking of the well-known double-gradient hypothesis of Runnström concerning the echinoderm egg. There we have a close resemblance to the phenomenon dealt with here.

If we cut out a narrow transverse section from the middle part of the planarian body, we always get a head anteriorly and a tail posteriorly; if we imagine the narrow section made so short as to vanish but still having neoblasts available, we should find that we had a cluster of totipotent cells in competition, the anterior half of them 'animalized', the posterior half 'vegetalized', and then the double gradient of Runnström would be at work.

Now, every comparison has its weak point, and this one too. In the planarian blastema the polarity is imposed by the remaining adult body; in the egg the polarity is imposed on the oocyte in a manner as yet unknown. But the analogy shows us, I think, that the polarity of the planarian body is a structure derived directly from the oocyte.

Here the time-graded field enters into the discussion.

The *Janus-head* or 'Janus-tail' has always puzzled morphogenetecists. We know that polarity may be reversed by external stimuli in hydroids: oxygen, pH, light, electric current, etc. But why should very short transverse segments from the forepart of the planarian body often regenerate heads at both anterior and posterior surfaces; and why should transverse segments from the hindpart often regenerate two tails?

It should be emphasized that only very short segments regenerate Janus-heads or Janus-tails. It should also be pointed out that Janus-heads are made only in the anterior part of the body, Janus-tails only in the posterior part, and neither of them in the mid-part. It must further be borne in mind that in an ordinary transverse segment from the forepart the head-blastema is the first to be differentiated, but in a posterior segment the tail-blastema. Therefore—again as in the echinoderm egg—the forepart is more 'head-minded', the posterior part more 'tail-minded'. This is probably due to the structure of the nervous system.

A very narrow transverse segment from the forepart contains a part of the time-graded field with a high head-building rate. The totipotent cells form the anterior and posterior blastemata almost equally fast. I suggest that the speed in the forepart of the time-graded field is so great that the totipotent neoblasts have had time to direct themselves into head-formation, also at the caudal wounds, before inhibition from the very small remaining part of adult tissue has got a chance to inhibit, the more so as such a narrow strip of tissue is nearly disarranged by the massive migration of the neoblasts. The same may of course be said—*mutatis mutandis*—of posterior narrow sections giving rise to Janus-tails. In mentioning the manifestation of polarity in the blastema I have not used such words as organization, organization forces, induction and the like. I must admit that I am not very enthusiastic about such notions. It seems strange to me that every level of the body should be able to 'organize' or 'induce' head-formation in an anterior blastema; and moreover that every level should be able to discern whether to make a head or a tail. It seems more probable to me that lack of proper inhibition gives the blastema its main direction of determination.

INHIBITION

Wolff and Lender (1950) and Lender (1951) have shown that the first structures to be regenerated in an anterior blastema are the head ganglia. Ribonucleic acids are conspicuous elements in the nerve cells; RNA is also a conspicuous element in the

neoblasts; they are strongly stained by pyronine and by Einarson's gallocyanine. It has been shown by my wife and myself that RNA accelerates head-regeneration in starved planarians. (A. and H. V. Brøndsted, 1953). All this opens up certain possibilities concerning the mechanism of morphogenesis in the planarian blastema. Now Brachet has proved that RNA accumulates in the dorsal part of the amphibian germ. This led me to suggest that the vertebrate germ may well be regarded as a time-graded morphogenetic field analogous with the regeneration blastema.

We know from a great number of experiments that a vast assortment of stimuli may evoke neurulation in the ventral epidermis of the young amphibian gastrulae; moreover, Barth (1941) has shown self-differentiation in explanted ventral ectoderm; this proves that the ventral epidermis has potencies of the same kind as the dorsal one. Could it be that normally it does not reveal these potencies because its rate of cytoplasmic reactivity is too slow, so that inhibition from the dorsal parts suppresses its tendency to differentiate? It may well be, in induction experiments, that contact with presumptive entomesoderm and with various metabolites accelerates its activities so as to reach so high a level of reactivity towards gene-action and hormones produced by gene-activity as to be able to escape inhibitory influences. This hypothesis might be tested experimentally in explants.

The current hypothesis, worked out by Holtfreter, Brachet and others, is that some sort of blockage exists in the ventral epidermis; if the blockage is removed then neurulation follows. I think this hypothesis is right, and I only suggest that the blockage is imposed on the ventral epidermis by the inhibitory influence from the far-advanced dorsal epithelium, the 'high-point'. This reasoning leads to a working hypothesis of a serological nature.

Long ago I suggested (Brøndsted, 1936) a mechanism for the sorting out of cell types in the reconstitution bodies of freshwater sponges pressed through bolting silk. Four main cell types could be discerned in the dissociated cell material. The results were confirmed shortly after by Brien (1937). The problem was to elucidate how the various cell types regained their proper situations so that a working system could be re-established.

While watching the movements of the isolated cells at the bottom of the dish I very often saw a curious phenomenon: when two cells met, then one or both might wriggle and send out pseudopodia at a far greater speed than when not in contact; the phenomenon was photographed by the time-lapse technique. Moreover, when two cells touched one another, one of them might throw out a pseudopodium with explosive force, as shown in Figure 20.

Here, surely, one might speak of negative cytotaxis. I therefore suggested that such movements, arising from mutual incompatibility between different cell types, might well be conceived as morphogenetic movements leading to reassortment. I regret that I have had no time to pursue this very promising problem. It was therefore with much pleasure that I found that Holtfreter later (1947) adopted similar views.

I think that this problem is connected with far-reaching lines of research concerning serological differences arising during early embryogenesis. I have not time here to go deeper into the matter. A very comprehensive and clear survey has been given by Woerdeman (1953).

I venture to suggest that serological incompatibility is at the root of negative cytotaxis. I further venture to suggest that when a cell or a group of cells during early morphogenesis has started determination in a fixed direction, it builds up a serological equipment characteristic of the cell or group of cells, and thereby exercises negative cytotaxis on neighbouring cells which have not yet reached a level enabling them to start differentiation; these neighbouring cells are inhibited from differentiating along the same lines. It should be possible to test this working hypothesis by the explantation technique.

According to this hypothesis inhibition is a question of serological nature; it is transmitted from cell surface to cell surface. The response of a cell to the stimulus

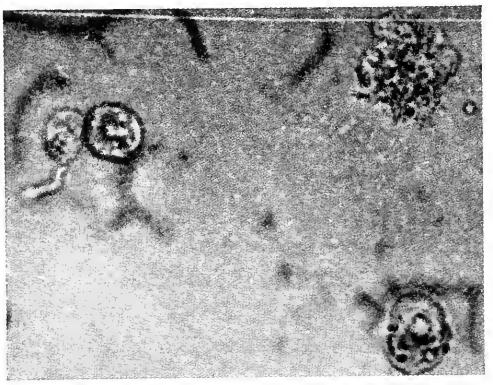

Figure 20. Spongilla lacustris. *Photomicrograph of two cells touching one another; one is giving off a pseudopodium with explosive force* (× 1,000). (*Brøndsted*, 1936.)

from its neighbour depends upon its reactive state. This in turn is determined by the situation of the cell in the time-graded morphogenetic field. And this consideration brings us to the concluding part of my paper.

THE RELATION BETWEEN GENE-ACTION AND THE TIME-GRADED MORPHOGENETIC FIELD

It has always puzzled embryologists that cells presumed to contain the same genome should differentiate differently. Even for this there is no conclusive proof, so long as it is not possible to cause differentiated cells to dedifferentiate into a totipotent condition from which they might then regenerate all parts of a new organism, including the germ cells. The same problem of differentiation exists both for embryos and for regenerating blastemata.

However it is difficult to transfer our notions of evocation and induction from embryogenesis to morphogenesis in the blastema. In an embryo provision is made for the unequal distribution of substances to the blastomeres, so that they are different from the start. On the other hand in the blastema all the cells are at first alike, and any induction or evocation must be initiated after the wound has been inflicted. It

is very difficult to imagine that head-inducing capacities should exist at several cell levels of the body when the wound faces forwards, but tail-inducing capacities at the same levels when the wound faces backwards.

It is not known what force imposes the time-graded field on the blastema, and drives the cells at the high-point to differentiate more quickly. However it is possible that crowding influences their metabolism, perhaps for instance through a deficiency of oxygen.

The blastema derives its polarity from the adult tissues. In the words of A. E. Needham (1952) concerning the determination of the blastema, 'There remain a number of morphogenetic difficulties, however, which cannot be explained by Child's simple theory, and will ultimately demand a more sophisticated scheme with more emphasis on qualitative gradients, and on multilateral competition, rather than on unilateral dominance—a democracy rather than a monarchy. The democracy is not one of anarchic equality, but one in which every section normally ensures its appropriate self-expression.'

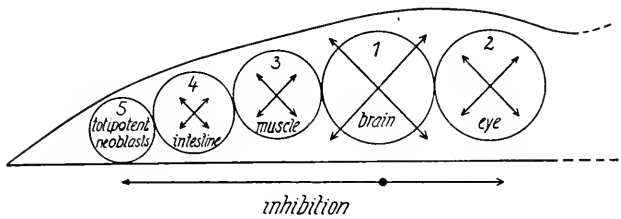

Figure 21. Schematic representation of cell clusters in a transverse section of a blastema in formation. Explanation in text.

It is now well established that the effects of genes become apparent—whether morphologically, physiologically or biochemically—successively in time. This has been shown by Hadorn (1948) and Poulson (1945) on lethal genes in *Drosophila.*

In the following working hypothesis, the differentiation of the blastema is interpreted in terms of successive gene actions and of inhibitions exercised by the median parts over the more peripheral parts of the blastema. Centrally placed neoblasts (1 in Figure 21) differentiate more quickly, that is to say, their cytoplasm more quickly reaches such a state as to respond to brain-determining gene actions. In differentiating they inhibit the neighbouring cluster of cells (group 2) from responding to brain-forming gene actions. The next gene action in time is eye-determining, and to this these adjacent cells now respond. Neoblasts later in the order of differentiation are inhibited from forming brain and eye, and so form muscle (group 3), intestine (group 4), or do not differentiate (group 5). These last remain totipotent. This hypothesis is represented schematically in Figure 22.

Geneticists are now laying more and more stress upon the conditions in the milieu necessary for the unfolding of gene action. Beadle and others have stressed the importance of the cytoplasmic state of single cells, and the work of Bonner (1952) and Sussmam (1952) on the slime mould *Dictyostelium* suggests the same principle of cytoplasmic reactivity. In this connexion we must clearly consider the conditions in the time-graded morphogenetic field.

Various obvious simplifications have been made in this discussion; interaction of genes has been left out of consideration; and those who study such phenomena as eye colour in *Drosophila* will stress the complexity of gene action and the influence of hormones. However, planarians are very primitive organisms, and are not so far removed from cell colonies as to make impossible the idea that in them genes act to a large extent directly upon the cytoplasm of the neoblasts.

The time-graded morphogenetic field and the gene actions are delicately balanced, and it is not surprising that irregularities should sometimes occur. For instance, occasionally the cell cluster to the left of the brain (group 3 in Figure 21) forms eye tissue instead of muscle. Several extra eyes may be produced under the influence of LiCl (Brøndsted, 1942). Waddington (1947, p. 47) has pointed out that 'during a

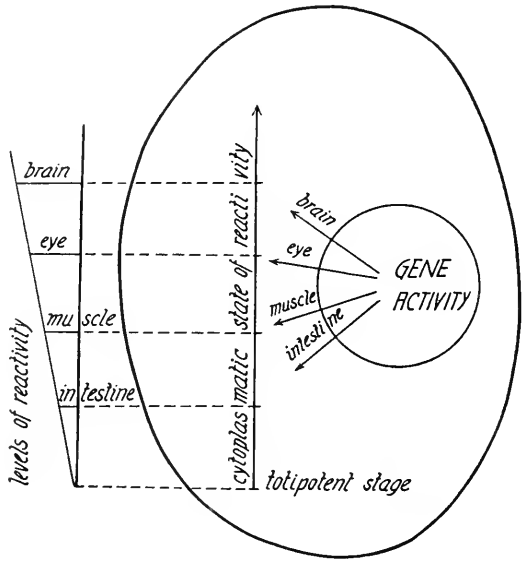

Figure 22. Schematic representation of the fate of a cell lying either in level 1, 2, 3, 4 or 5 of the time-graded field as set forth in Figure 21.

period of competence there may be more than two alternatives open to a tissue'. The heteromorphoses reported by Waddington (1947) affecting the antenna of *Drosophila* and the well-known cases in Crustacea and primitive insects affecting leg, eye-stalk, and antennal regeneration, may be due to a similar lack of adjustment, although in these higher organisms hormones probably play a major role in the process of differentiation.

The postulated time-graded field may also break down in the egg. At least I am strongly inclined to interpret Witschi's (1922, 1952) well-known experiments with teratomes arising from over-ripe eggs as a breakdown of the field.

It would be of great interest to grow an isolated blastema, and see what it produces. So far this has not been possible, because it has always cytolysed.

In further investigations we are especially interested in biochemical aspects of regeneration, including amino-acids in various parts of the blastema, and the effects

of vitamins on differentiation, with special reference to the connexion between RNA, amino-acid metabolism, and protein synthesis. There is much evidence, summarized by Needham (1952), that in nerve cells morphogenesis is linked with RNA content and protein synthesis. It might be suggested that gene activity directs protein synthesis when this has reached certain levels, different for the different cell clusters in the time-graded blastema.

In conclusion, I stress the need for biochemical studies of the kinetics and time relations of enzymatic processes concerned in morphogenesis, and of morphogenetic studies of the time relations of the differentiation of totipotent cells from different parts of the embryo, such as we are trying to make on different parts of the time-graded morphogenetic field.

SUMMARY

Regeneration experiments with planarians have revealed the existence of a time-graded regeneration field. It has been shown that in the regeneration blastema there exists a median high-point, or rather a right and left one close to the mid-line, which starts earliest in making head structures. It has been shown that inhibiting forces emanating from the high-point prevent lateral parts of the blastema from differentiating into ganglia and eyes, thereby securing a harmonious regeneration.

It has been made probable that the problem of bilaterality may be understood on the basis of the time-graded field. It is suggested that polarity is in some way connected with inhibition and the time-graded field.

It is suggested that inhibition is a chemical mechanism of a serological nature producing inability in neighbouring cells to reach the same cytoplasmic reaction level as that of the cell from which the inhibition emanates.

It is suggested that the time course of inhibition is correlated with the temporal succession of gene-activity.

It is suggested that RNA plays a major role in the setting up of the time-graded regeneration field and in doing so is the basis of the time-dependent processes in the blastema.

Some suggestions are made for experimental procedures to test the working hypothesis here set forth.

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Discussion

OF PAPERS BY (1) C. H. WADDINGTON AND (2) H. V. BRONDSTED

Chairman: E. Zeuthen

C. D. Darlington. With regard to the classification of genetic particles, it is important to remember that the biological distinctions between virus and plasmagene (gene-initiated or otherwise) break down at the chemical level. The most recent evidence is especially significant. Kenneth Smith (1952, *Biol. Rev.* **27**, 347–357) has found that cell constituents of the sugar-beet at a certain stage of its development propagate themselves without limit in the cow-pea. They behave like a virus, or provirus I should say, as infection is artificial. Thus a particle or a chemical entity determined by heredity can in the course of development become the means of infection.

E. W. Yemm. The sugar-beet and cow-pea virus described by Professor Darlington seems to imply a transmission of autonomous particles, capable of protein synthesis, from one species to another. In this connexion it is of interest that cellular proteins from leaves of different species are very similar with regard to their constituent amino-acids; we have recently shown that the similarity extends to the cytoplasmic and chloroplastic proteins of the same species. It follows that the amino-acids required for synthesis of cytoplasmic proteins may not vary very much from species to species, and this may in part account for the possibility of interspecific transmission of the type discussed. It is possible that variation of infectivity depends on the activity of the leaf in protein synthesis at different stages of development.

C. H. Waddington. The example of the cow-pea virus shows that certain embryonic proteins, when transplanted so as to become associated with a foreign nucleus, can multiply in a more or less uncontrolled manner. I agree with Darlington that this implies that the problems of normal cellular differentiation and of pathological virus-like infections belong to the same general area of developmental-genetical discourse. But it is to my mind much less clear that there is any very close parallel between the processes which bring about the orderly development of locally differentiated regions in an embryo and those which underlie the usually pervasive and uncontrolled alterations of cell type caused by a virus infection.

L. Rinaldini. The narrowing down of potentialities that accompanies differentiation suggests a progressive loss of synthetic properties coupled with the appearance of new specialized functions. This loss, if genetically determined, may mean that some genes are systematically inhibited during embryogeny. In Professor Waddington's scheme of cyclic reactions the reaction products (P) would on mass active considerations be expected to depress the formation of their precursors, but if P were removed from the cell the reaction would be accelerated, and a type of cell would be obtained which would produce more and more P. By cell division more cells with identical metabolism could be produced, but the excess P shed into the surroundings would inhibit cells from developing in the same direction. In this way we have two parallel

mechanisms combined, one for preserving identity and the other for ensuring diversity. By such a mechanism some of the experimental results obtained in planarians by Professor Brondsted, such as double gradients, space-time-graded inhibition, and blockage, might conceivably be explained. It might also offer a new view point on the problem of unspecific inductions, as the unspecific stimulus would simply have the effect of removing or destroying the inhibitor. These views are put forward in a purely tentative way, and no doubt many alternative explanations could be sought.

C. H. Waddington. I agree with Dr. Rinaldini that it is quite possible that differentiating cells produce diffusible substances which inhibit neighbouring cells from entering the same path of differentiation. A hypothesis of this kind has recently been proposed by Dr. Merrill Rose. However, although possible, it is not in my opinion very probable that such inhibitory substances play an important part in early determination, though they may more probably do so in the growth of already differentiated tissues at later stages. In the early stages of development cells differentiating in one way may stimulate their neighbours to differentiate in the same way (cf. 'homoiogenetic induction'). It may be mentioned that only if the substance P acts autocatalytically, to encourage the production of more P, does one obtain an exaggeration of an initial difference in conditions; this would not happen on ordinary mass action principles.

Synchronous divisions in mass cultures of the ciliate protozoon Tetrahymena pyriformis, *as induced by temperature changes*[1]

by

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INTRODUCTION

NATURE has supplied us with a few cases in which neighbouring cells in a cell community are in phase so that they more or less simultaneously pass through the only recognizable part of the cell cycle, namely cell division. Stimulating examples are presented in d'Arcy Thompson's (1917 and 1942) book, and in the review of Sonnenblick (1950) dealing with early embryonic stages in the insect egg. The fertilized insect egg, like many other syncytia and plasmodia, shows a perfect synchronization of nuclear division for many generations, and, as a consequence, the first cell division which cuts out the blastoderm of about 2,000 cells is synchronous in the whole embryo. Apparently, chemical situations identical throughout a syncytium or a plasmodium induce nuclear and cell division. In cell suspensions cell borders may act as barriers to the diffusion of controlling agents; and in tissues where cells occupy relatively fixed positions in a medium which is not mixed, distance alone would prevent chemical interaction between any considerable number of cells.

Whenever synchrony of division is observed in a tissue it is therefore limited to groups of cells all within a short distance of each other. This observation is known to many workers in histology and tissue culture. It may be significant that in spermatogenic tissues where cells line a lumen in which free diffusion and even some mixing may take place, we have the prettiest cases—still limited to a small mass of cells—of synchronous growth and division.

THE GOAL AND THE WORKING HYPOTHESIS

For the reasons mentioned above we are in a difficult position when it is a question of relating growth to the basic phenomena of synthesis and multiplication in the cell cycle. The goal we had in mind was therefore that of establishing a synchronized system in which the cell cycle could be studied on aliquots representing successive stages in the cycle. Such a system would serve as a useful substitute to the few already known, such as egg material and plant spores (Erickson, 1948). The introductory

[1] This work was supported by grants from Rask-Ørsted Fondet and from the Eli Lilly Foundation.

remarks clearly indicate the possibility of inducing synchronous cell divisions by controlling the chemistry of the medium. Nevertheless, in view of our present ignorance as to the best way of making such an approach, we decided on changes of temperature as a possible way of changing the relative concentration in the cells of a great number of metabolic intermediaries, which might include one or more agents necessary to enable the cell to pass from one phase of the cell cycle to the next.

If in a mass culture of a micro-organism there is a random distribution of all stages in the cell cycle, we shall find that as long as the organism is growing exponentially a constant fraction of the cells will be in division. We observe a steady state in which there is an equilibrium between cells entering and cells leaving the division stage. Transitory changes in the number of dividing cells divided by the total number of cells, termed hereafter the 'division index', may indicate either that for some reason the time which the cells spend in division changes relative to the time occupied by the whole cycle, or that cells have accumulated into groups which tend to divide together and possibly pass through one or more full cycles together.

In our search for group formation we have accepted the division index as our guide, division indicating all stages from onset of furrowing until separation of the two cells. Decision between the two possible interpretations can only be made if multiplication is estimated by actual cell counts.

Let us then accept as a working hypothesis that some phase in the cell cycle has a higher temperature coefficient than any other. Indeed, Ephrussi's work (1926) on dividing sea-urchin eggs had indicated a case where this is so. Such a phase will be more sensitive to temperature changes than any other. With lowering of the temperature cells in this phase will tend to drop behind and unite with cells behind them in the cycle, whereas with increase in temperature they will tend to catch up with cells in front. In both cases we should get group formation, detectable only at the time when the group passes through the division stage. If experiments were to confirm our working hypothesis thus far, the next step would then be to transfer the culture back to the first temperature, after a time shorter than the duration of the cell cycle at the new temperature. When our group was again in the sensitive phase the temperature would again be shifted. For every such periodic change in temperature the original synchronized group should become larger, and in the end the whole culture should become synchronized. The first part of our working hypothesis was confirmed by experiment; the second was never put to serious test. It is mentioned here because a number of our experiments might otherwise seem curious.

THE EXPERIMENTAL ORGANISM

For our experimental organism we chose the ciliate protozoon *Tetrahymena pyriformis*, because it grows readily on dissolved and fully defined media, as reported by Kidder and Dewey (1951). So far, however, we have grown our cells in 2 per cent. proteose peptone (Difco) plus 1 per mil. liver extract (Wilson Lab.) with salts as in Kidder and Dewey's synthetic medium *A*. The cultures were shaken and aerated with a flow of air which was passed over them. Each culture contained 150 ml. of medium and the total synchronized population represented about 100 mg. wet weight of cells.

Another advantage of *Tetrahymena* is that the cell cycle is so short ($2\frac{1}{4}$–$2\frac{1}{2}$ hours at the optimum temperature of 28–29° C.) that several cycles can be studied in succession in the course of a day. Also the cell is reasonably big, being 50–70 microns in length. Some will consider it a serious limitation that the cytology is relatively little known and furthermore that it is atypical of cells in general. As in other ciliates there is an amitotic macronuclear and a mitotic micronuclear system. Our cells (the Lwoff strain), like other strains of *Tetrahymena* which have been grown in pure culture for a long time, do not possess a micronucleus and are therefore amitotic and asexual. Recently, however, mating types of *Tetrahymena*, all of them having a micronucleus of course, were isolated by Elliott and Gruchy (1952) and were further described by Elliott and Hayes (1953).

Chromosomes have been demonstrated in the dividing micronucleus of *Tetrahymena*, as in other ciliate micronuclei (Elliott and Hayes, 1953; Fauré-Fremiet, 1953; Sonneborn, 1949). In the macronucleus of ciliates a ribonucleic-deoxyribonucleic acid system is present, as in other kinds of cells, but morphologically it is organized in a very special way. In the macronucleus of the ciliates clearcut volume changes (Popoff, 1908) and structural changes, simultaneous with the division of the micronucleus and thus preceding cell division, can be revealed by the use of nucleic acid stains (Fauré-Fremiet, 1953; Sonneborn, 1949).

CHARACTERIZATION OF STAGES

The successive stages in the cell cycle may also be characterized by other means in addition to the standard cytological ones, for instance by possible differences in the capacity of the system for growth. A single stationary phase *Tetrahymena* cell was introduced into a microrespirometer and the rate of respiration was accepted as a measure of the respiring mass present at any time. The cell synthesized and multiplied in the respirometer. The results of a few runs (Zeuthen, 1953a) are shown in Figure 1. From the onset of furrowing in one division until 10–20 minutes before the next division there is a linear increase in mass ($\sim$ respiratory rate). In the account which follows this period will be called the 'synthetic phase'. Then follows a period lasting 10–20 minutes with no further increase in mass. This will be called the 'pre-division period'. When division of the cell begins the macronucleus is already somewhat stretched. A study of the literature on ciliates in general and on *Tetrahymena* in particular makes it exceedingly likely that the 'pre-division period' covers or partly coincides with the period of macronuclear swelling and reorganization referred to above. At the end of the pre-division period, or at the beginning of division, synthesis is resumed, and at twice the rate before 'pre-division'. Thus, on the basis of the curves given in Figure 1, and for purely practical reasons, we have divided the cell cycle in *Tetrahymena*, growing on our media, into three phases: *cell division* coincides with the early part of the *synthetic phase*, and in the *pre-division* period there is a block to further rise in respiration. In support of this way of dividing the ciliate cell cycle into physiological phases, reference is made to a number of papers in which an attempt is made to follow cell volume from one division to the next. The situation is reviewed by Richards (1941) and by Wichterman (1953). In the present writer's opinion the overall picture given in their papers is very suggestive of that observed

in *Tetrahymena*, with rate of respiration used as a measure of mass. The paper by Chalkley (1931) demonstrates that in *Amoeba proteus* (which divides by mitosis) the percentage rate of synthesis decreases as the cell approaches division. Therefore,

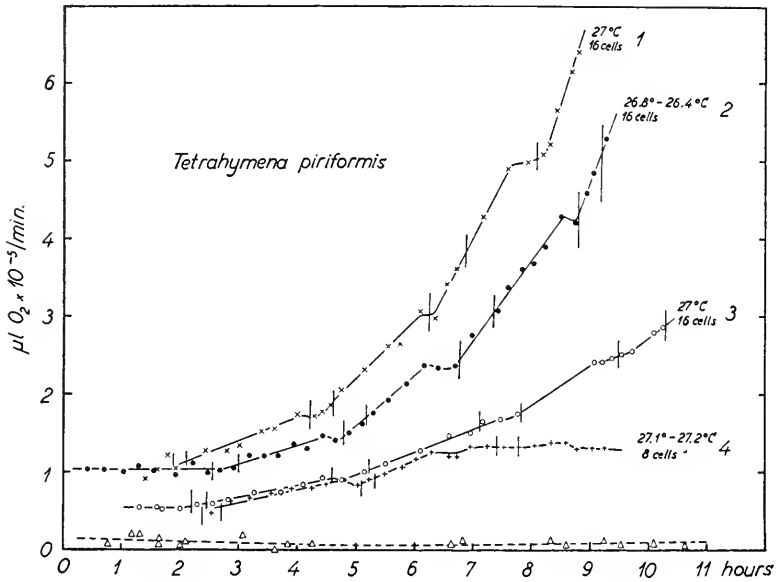


Figure 1. *Rate of respiration plotted on an equidistant scale against hours. Of each pair of vertical lines intersecting the curves, the first one indicates onset of division, and the second indicates that the daughter cells have separated. All experiments start with* 1 *cell; the number of cells recovered at the end of the experiments is indicated. In experiment* 3 *the log phase is over after division* 3, *in experiment* 4 *after division* 2.

with division, the rate of synthesis must be stepped up, more or less as in *Tetrahymena*. However, these data are not good enough for demonstrating a possible block to synthesis prior to division.

TEMPERATURE SHIFTS BELOW OPTIMUM

First the effect of temperature shifts from optimum or sub-optimum to a low temperature (7° C.) were investigated, with the results indicated in Figure 2. Multiplication continues at the low temperature. Three different experiments are put together. The first (to the left) shows the scatter of the division index in a normal culture kept at 29° C. The second indicates that the division index tends to drop slightly and continuously (perhaps with a lag) after transfer to the low temperature. This only tells us that in the cold fewer cells enter into, than out of, division. After return to the high temperature (third experiment, 15 hours at 7° C.) the culture is first completely emptied of cells in division but after 1½ hours a group representing about 30 per cent. of the whole population enters into division. However, if the high temperature is maintained there is no evidence of this group's reappearing after the

lapse of another cycle; it merges completely with the rest of the population. With this same culture two more bursts of divisions, both of about 25 per cent., were produced as a consequence of cooling to 7° C. for 2 hr. and 16 hr. respectively. Thus, within the limits studied, the length of the cooling period is of no significance for the size of the 'burst' obtained. From a later study of the highly interesting paper by Popoff (1908) we have found a possible explanation. Popoff carefully measured cellular and macronuclear volume in three different ciliates at different temperatures of growth. Transfer from 25° C. to 10° C. or 14° C. resulted in readjustment of body size and of macronuclear volume. Probably after less than a cell cycle at the low

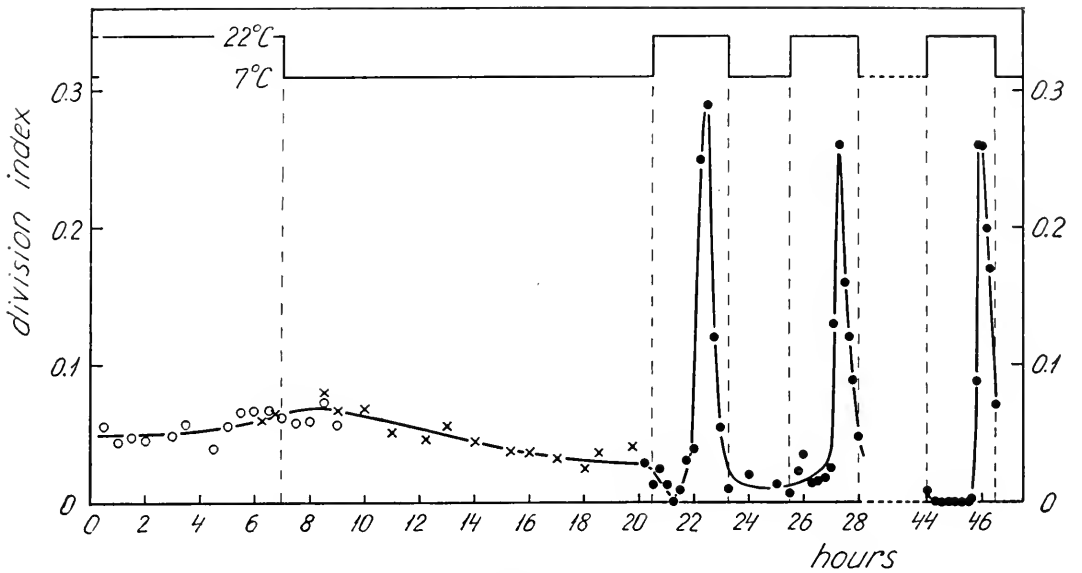


Figure 2.

temperature both increased about 2 times in size, although the macronucleus relatively more than the whole cell. No return was made to 25° C. Had it been it might have resulted in readjustment to the smaller cell size, and thus to increased division activity. Furthermore, this activity could be expected to be independent of the length of stay in the cold if only the cells had been exposed long enough to assume the large size typical of low temperature.

TEMPERATURE SHIFTS ABOVE OPTIMUM

In *Tetrahymena* we have obtained the best results by shifting the temperature between optimum (or, in early experiments sub-optimum) and temperatures 3–5° C. above optimum. In our first experiment (transfer from 24° C. to 32° C. for 3½ hr. with subsequent return to constant 24° C.) we obtained the formation of a group corresponding to 34 per cent. of the whole population, and the appearance of a small second peak on the curve supplies evidence of continued cycling at constant optimum

or sub-optimum temperature (Figure 3). Acting on the theory on which we started out, in a second experiment we subjected the culture to another temperature increase

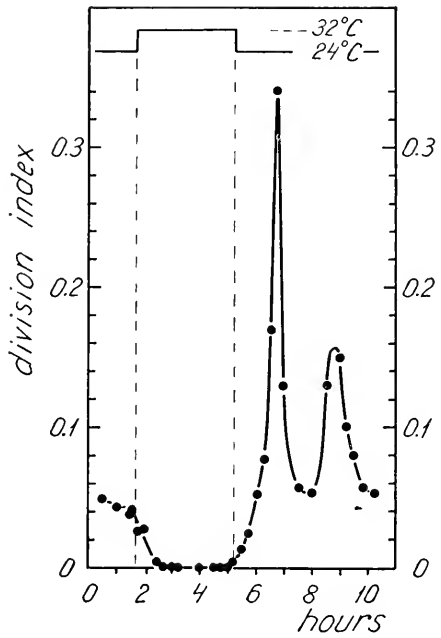


Figure 3.

in order to eliminate the second peak and let it unite with other cells to form a bigger group. We were not completely successful, as illustrated in Figure 4. We induced a second cycling, but did not as planned eliminate the small peak. Apparently the

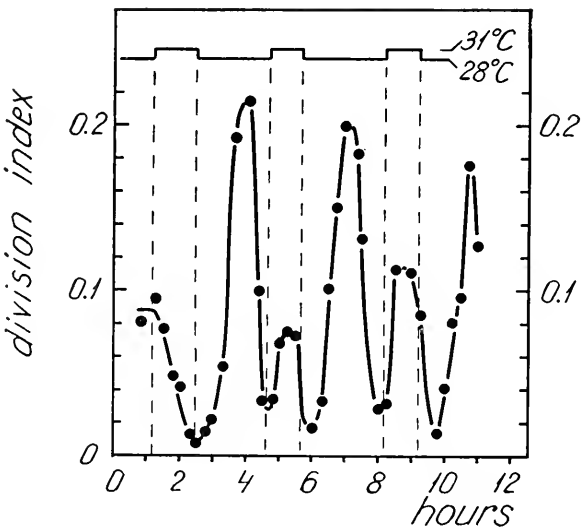


Figure 4.

second temperature rise should have come still earlier, and we then realized that it would be much better if we did not let even the first peak develop. Instead we supplied the stimulus for it by one short heat shock, but did not let it develop because the culture was subjected to another shock before it could do so. Hoping to concentrate

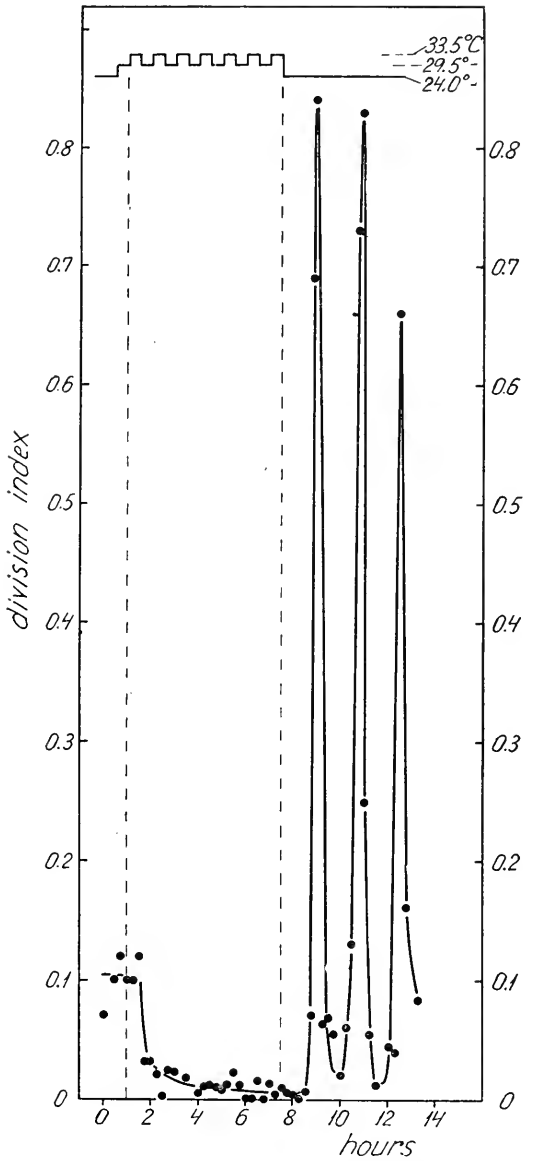


Figure 5. Standard intermittent heat treatment produces cycling in the culture after return to optimum or to room temperature. In this as in other graphs the temperature curve shows the switches of the control clock. In the culture the temperature fluctuates with the amplitude indicated, but more nearly according to a sine curve.

the whole culture into one group, we applied a series of heat shocks (33·5° C.) each of half an hour with half an hour at optimum temperature (29·5° C.) in between. This had the expected effect, as illustrated in Figure 5. During the intermittent heat treatment cell divisions were continuously suppressed, but after return to 24° C. the vast majority (85 per cent.) of all cells entered into division simultaneously. As two more peaks of synchronous division were observed at 24° C. this group must have passed through a total of three cell cycles at constant room temperature. This experiment was repeated many times, always with the same result. It was of no significance whether the cells were returned to constant optimum or to constant room temperature, and we got more or less the same result whether we let the temperature cycle between

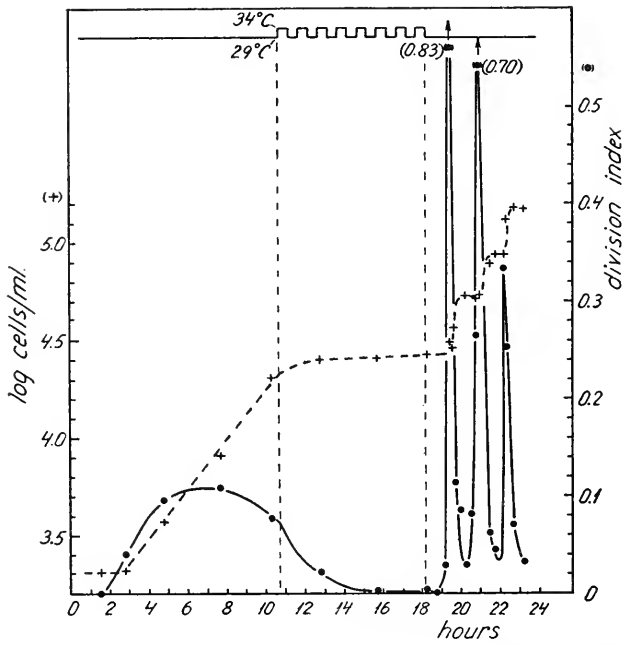


Figure 6. The continuous curve represents division index, and the broken curve represents cell counts.

28° C. and 34° C. or between 29·5° C. and 32° C. Besides the well-synchronized cycling there is one more striking thing to observe from Figure 5, namely, that the intervals of time between the peaks represent only about 60 per cent. of the duration of the cycle in normal cells. Whereas cell division was blocked during heat treatment, after end of treatment it went on at a faster rate than usual.

These preliminary results were checked with actual cell counts as demonstrated in Figure 6. Multiplication was blocked during the period of cycling temperature, and after the return to optimum temperature the cell count increased in the expected step-wise manner, the height of every step approximately corresponding to a doubling in number. The division index was followed in the same experiment. Three successive bursts of divisions were observed.

SYNTHESIS IN SYNCHRONIZED CULTURES

The rate of synthesis was estimated from the total cell volume in aliquots. One-ml. centrifuge tubes in which the bottom was made into a uniform capillary were used. The cells were densely packed into the capillary by centrifugal force and cell volume was measured as the height of the column of cells, assuming complete packing in all cases. In Figure 7 the cell volume per unit volume of culture is plotted on a log scale together with the cell count. During heat treatment there is little if any multiplication, but cell volume is increased to three times what it was. However, after the end

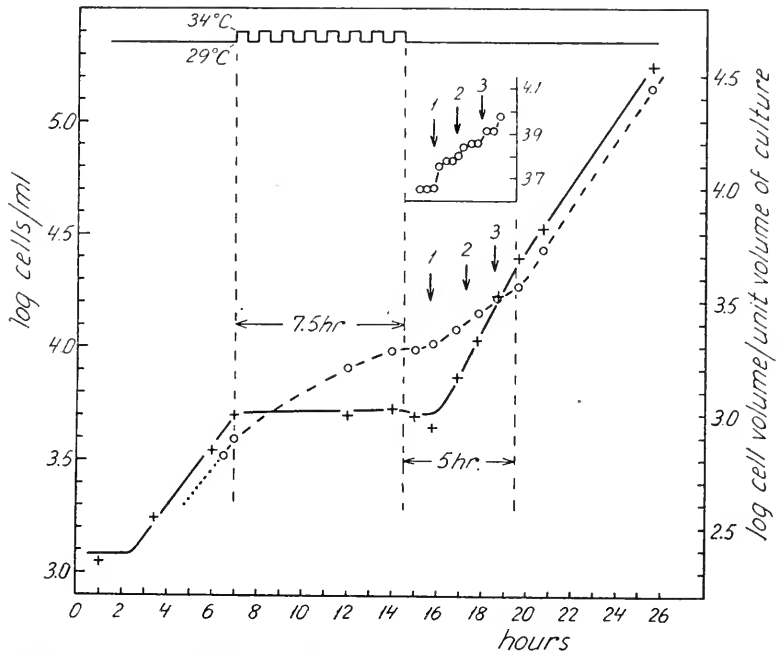


Figure 7. The continuous curve represents cell counts; the broken curve represents volume of cells per unit volume of culture.

of treatment multiplication sets in at a rate faster than in the untreated culture. (In this case no attempt was made to resolve multiplication into the steps in which it occurs.) Synthesis continues however at a slower rate than in the untreated culture. After 5 hours the multiplication rate decreases and synthesis speeds up, both to the rate before treatment. The relative position of the two curves 5 hours after end of treatment, as compared with the situation before heat treatment, indicates that normal cell size has become re-established. In the case presented in Figure 7 the culture was diluted after the end of heat treatment. Otherwise it is not possible in one experiment to demonstrate these effects, particularly the return to the normal rate of synthesis and of multiplication many hours after end of treatment. We want to emphasize that the dip in cell count after dilution is atypical and may be due to an error made in the dilution. If this is correct the absence of synthesis in the first half-hour after dilution may also be erroneous. The inverse relationship between rate of synthesis and

rate of multiplication in the hours after heat treatment was easily demonstrated even without dilution.

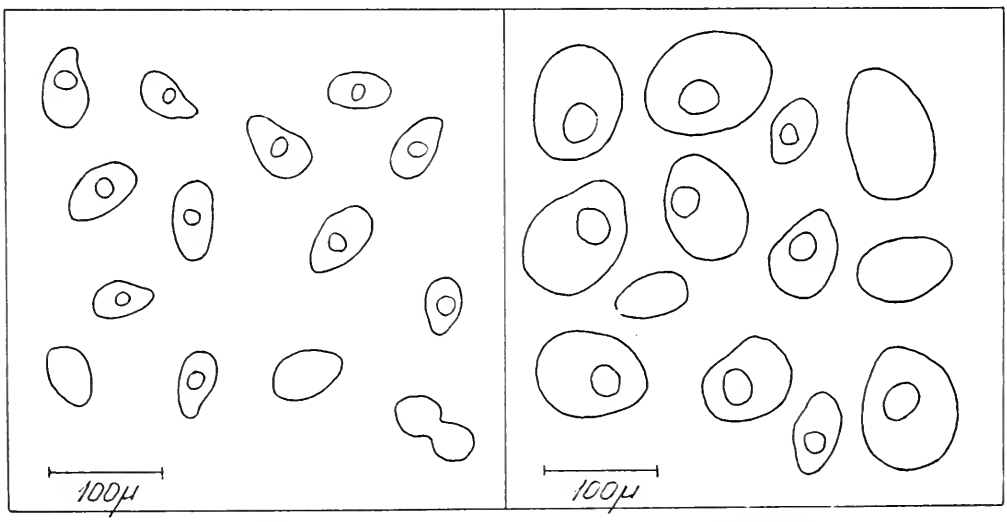
Dr. Hoff-Jorgensen kindly made some DNA determinations for us using the method he is describing in this symposium. The data are included in Table I together with

TABLE I

Time, hours	Cells/ml.	Total volume of cells (μl.) per ml. of medium	Average cell volume (μl. $\times 10^{-5}$)	DNA (μg.) in washed cells from 1 ml.	DNA (μg.) per μl. cells*	DNA per cell (μg. $\times 10^{-5}$)
0	32200	0·69	2·14	0·48	0·70	1·5
$\frac{1}{4}$	Heat treatment begins					
$5\frac{1}{4}$	48000	1·91	3·98	1·86	0·97	3·9
$7\frac{1}{4}$	45600	3·18	6·97	2·73	0·86	6·0
$7\frac{3}{4}$	Heat treatment ends					
$10\frac{1}{4}$	150000	4·13	2·75	5·08	1·23	3·4
$12\frac{1}{2}$	279000	7·43	2·66	7·18	0·98	2·6
$13\frac{3}{4}$	558000	8·91	1·60	9·08	1·02	1·6

* DNA (μg.) per μl. medium: 0·01–0·015.

cell counts and measurements of total cell volume per unit volume of the culture. The culture medium itself contains some DNA, but in concentrations of only 1–2 per cent. of what is found in the cells. We consider it most likely that the DNA in the cells represents synthesis and not just accumulation from the medium.



(*a*) *normal* (*b*) *heat-treated*

Figure 8.

In Table I is given the DNA per total cell volume in the aliquots, the DNA per cell, and also the DNA per unit volume of cells. It will be observed that total DNA follows total cell volume rather closely throughout. This means that while the cells grow bigger during heat treatment there is a more or less proportional increase in the DNA content per cell. After heat treatment multiplication outweighs synthesis, but the volume per cell and the DNA per cell decrease proportionately. The corresponding morphological changes are indicated in Figure 8. Normal (*a*) and heat-treated cells (*b*) were compressed to a standard thickness of 10 microns and photographed through the phase-contrast microscope. The outline of the cell body and of the macronucleus are shown as tracings. It is observed that the relative increase in size is about the same for the macronucleus as for the whole cell. It is therefore reasonable to assume that DNA is accumulating in the macronucleus of the heat-treated cell just in the same way as other specific cell components are accumulating in their natural loci. The situation is comparable to the state of polyploidy in cells dividing by mitosis.

RESOLUTION OF GROWTH IN THE SYNCHRONIZED CULTURES INTO STEPS

In Figure 7 an insert demonstrates that in all probability growth in cell volume increases more steeply after pre-division than before, thus confirming what is known for single cells of other ciliates (cf. p. 143). Respiration in the synchronized cultures follows a rhythmic pattern (Figure 9, open and full circles). As in the single untreated cells (Figure 1), the rate of respiration increases for some time during and after division, but it remains level for some time before division. (Only one division [the third in the experiment represented with solid circles] is not in agreement, out of a total of ten divisions observed in the three experiments on synchronized cultures.)

If we compare Figures 9 and 1 it seems necessary to conclude that the simple reason for the observed inverse relationship between synthesis and multiplication in the synchronized cultures (Figure 7) is that in the treated cells the synthetic phase is shorter than in normal cells, whereas the non-synthetic pre-division phase is unchanged, or perhaps even somewhat extended. Another fact to be observed in Figure 9 is that there is no clear-cut tendency for the rate of synthesis to accelerate throughout an experiment. This is very different from what was found in cultures started in respirometers with one untreated cell. As discussed before (Zeuthen, 1953) the curves of Figure 1 can be interpreted as indicating that in the synthetic phase the rate of growth is controlled by units rather than by the increasing amount of material accumulating in each cell. In the pre-division stage the units ('synthetic centres') double but they do not at the same time permit synthesis in the whole cell to occur. Synthesis is resumed only after the 'synthetic centres' have doubled, and they then control synthesis at a double rate.

Why did we not also find the same situation in the heat-treated cells which divide fast after the end of treatment? Perhaps because the big cell among other things is overloaded also with 'centres' controlling the rate of synthesis; these 'centres', like the rest of the cell, may increase in numbers but by no means double at every pre-division.

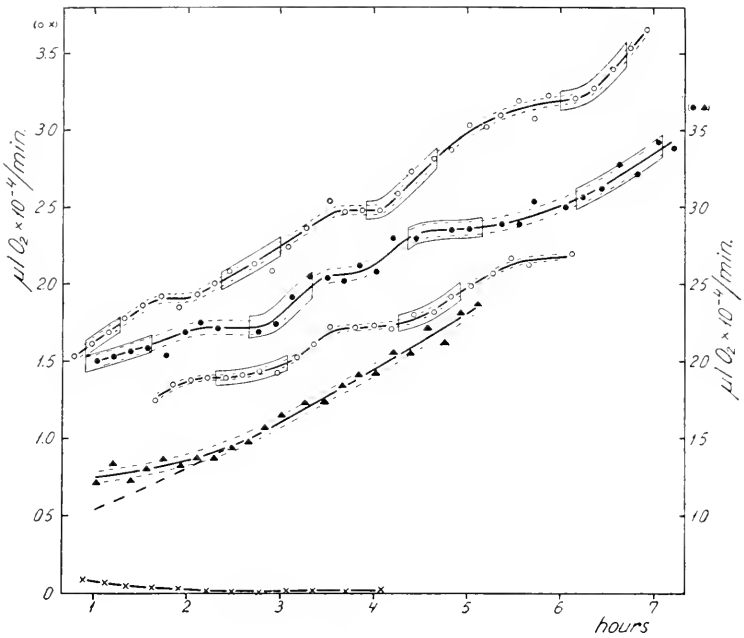


Figure 9. *Respiration in populations of* Tetrahymena *cells plotted on arithmetical scales against time in hours. Open and full circles: three experiments with synchronized populations. Upper curve:* 10 *cells initially present. about* 100 *cells after the experiment. Second curve from above:* 11 *cells initially present. Third curve from above:* 9 *cells initially present,* 70 *in the end. In the three curves frames indicate division periods observed in the divers. All curves are fitted by eye and in each case two broken lines represent* $\pm\sqrt{\frac{\Sigma d^2}{n-1}}$. *The fourth curve (triangles) is a control run with* 20 *cells from an untreated population. A control run with empty diver is represented by crosses. (Experiments by Mr. H. Thormar, method of Zeuthen* (1953).)

DISCUSSION

It is now time to consider what we actually do to the cells, when after growth at optimum or sub-optimum temperature we transfer them for the first time to the cold (7° C.) or to a supra-optimal temperature. In both cases the division index tends to drop after a time which is far shorter than the duration of the cell cycle at the new temperature. This shows that in both cases the rate of entry of cells into division is slower than that of exit from it. Apparently chemical processes occurring in the pre-division stage are exceedingly sensitive to temperature and have a narrower range of optimum temperature, with sharper decline on both sides, than processes underlying other phases of the cycle. We might also say that they have relatively high temperature coefficients below optimum and relatively low above optimum. For this reason the chemical disturbances of cells exposed to cold or to heat might be related to one another, consisting perhaps of changes in the relative concentration of metabolites essential for cells to enter a division. Also recovery from exposure to cold or

to heat follows the same pattern; cells prevented from dividing enter division with a delay which is much the same when measured at 22–24° C., irrespective of whether the cells have been exposed to low or to high temperatures (cf. Figures 2, 3, 4 and 5). The recovery time (90 ± 10 min.) at 28–29° C. is three times as long as the interval between the individual heat shocks in our standard treatment. That is why cells never divide during treatment. However, they do synthesize, which they would not have done, or would have done exceedingly slowly, at continuous high temperature. This suggests that a high rate of synthesis is resumed very quickly after return from high to optimum temperature. Thus, the success of heat treatment seems to depend on this different rate of recovery of independent mechanisms for growth and for control of the entrance, first into pre-division, then into division.

One more observation needs comment: even though apparently cold and heat block the cell cycle at some point prior to division, after return to constant optimum or room temperature the cells are in a stage which in time is long separated from the next division. This time is only slightly shorter than the duration of the cell cycle in the heat-treated cells (cf. Figure 5). Thus heat treatment, and perhaps also cold, may strike cells in a chemical situation typical of pre-division and leave them in a situation more typical of post-division. One reasonable suggestion is that intermittent heat treatment keeps cells in a chemical condition resembling that in a newly divided cell in which we may imagine that 'precursors of division' are minimal and the capacity for growth is high. Another possibility is that during heat treatment the cells pass through several incomplete cycles, by-passing the division stage. The latter situation would resemble the one produced by colchicin and radiation in mitotic cells.

A problem with which we are confronted and which is open for research with synchronized cultures is this: how do cells which have accumulated in themselves enough material for about 4 cells behave when an artificial block to division is suddenly removed? First of all, with the first division they do not spring directly into 4 cells, although very occasionally this has been observed. They divide in an orderly manner, first into 2, then into 4, and since synthesis occurs, divisions at the high after-treatment rate continue still further before normal cell size is re-established. The rate of synthesis after the end of treatment continues to be lower (about 1/2) than normal until normal cell size has become re-established. Thus, in *Tetrahymena*, we observe competition between synthesis and cell division. The fertilized egg represents an extreme case of such competition. In the ovary there is no division, only synthesis; however, fertilization removes a block to cell division, and divisions then follow each other in extremely rapid succession, while synthesis is completely suppressed during cleavage (cf. discussion by Zeuthen (1953b) and by Hoff-Jørgensen in this symposium).

Thus, in this competitive interaction between tendencies for synthesis and for division we find the giant *Tetrahymena* cells produced in heat treatment to be intermediate between dividing eggs and log-phase cells. If we include also the ovarian, rapidly synthesizing, non-dividing egg we have a series which represents all situations from complete ascendancy of the tendency to divide to absolute dominance of the synthetic machinery. In its normal cell cycle every growing and dividing cell switches to and fro between these two extremes.

By this time it will be painfully clear that we are not in the position to look back and point out exactly where we were right and where wrong in our working hypotheses. The essential thing is that we got some of the results (Scherbaum and Zeuthen, 1954) we were hoping for. In the synchronized cultures all those three phases are present into which we divided the cell cycle in normal *Tetrahymena* cells; only their quantitative relationships are somewhat changed. For bacteria, Hotchkiss (1954) has quite independently and almost at the same time worked out a method of synchronization based on very similar arguments and using temperature shifts. We have not made further reference to this work because this will be done by Dr. Maaloe (Maaloe and Lark, 1954), who is going to speak next.

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Plate 1 (*a*)

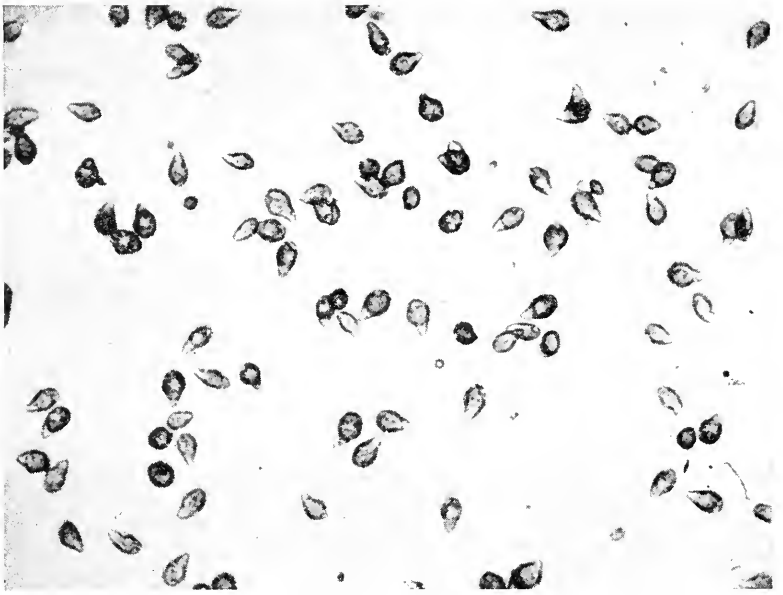

Plate 1 (*b*)

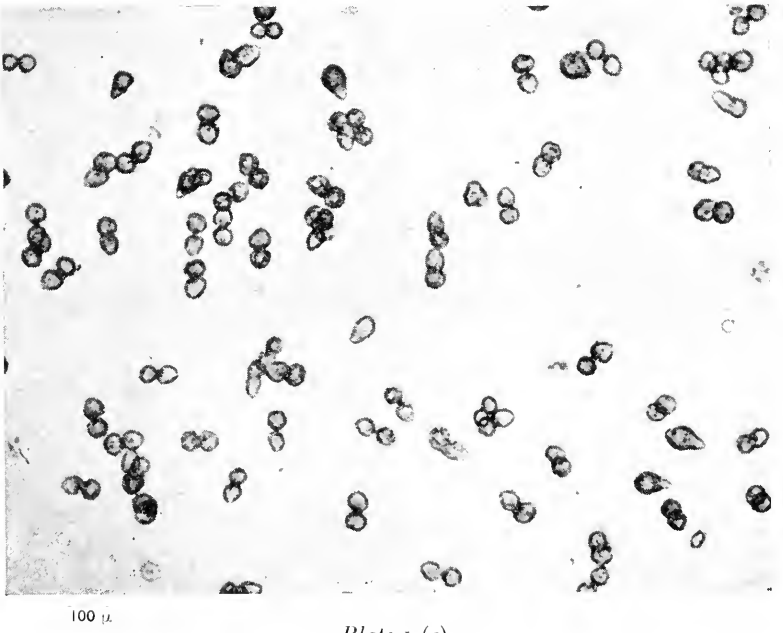

Plate 1 (*c*)

Plate 1. (*a*) *Untreated* Tetrahymena *cells from a log-phase culture;* (*b*) *after* 9 *hours of treatment with intermittent heat shocks;* (*c*) 90 *minutes after return to constant optimum temperature.*

Discussion

Chairman: J. F. Danielli

Muriel Robertson. In the protozoological laboratory at the Lister Institute we have repeated Dr. Zeuthen's work on the synchronization of cultures of the ciliate *Tetrahymena pyriformis* (W) with complete success. The variations in temperature must be repeated an adequate number of times, and thereafter the organisms are kept at the optimum of 28° C. rather than at 24° C. The nuclear division of the *Tetrahymena* is being studied. There is no micronucleus in this strain. The macronucleus does undoubtedly increase in size as compared with the untreated ciliates, but it does not at present appear that the number of chromosomes is increased.

B. F. Folkes. These results suggest a very sharp temperature optimum for the synthesis of some metabolic precursor required for the pre-division stage. This implies not only a high energy of activation for these synthetic reactions but also one of two possibilities: (*a*) an inactivation of the responsible enzymes at temperatures above the optimum, *or* (*b*) the presence of some other reaction with an even higher energy of activation which is competing for the same substrates or removing the reaction products. If we could distinguish between these possibilities we might be nearer the identification of the metabolic precursor. In the case of reaction (*a*), holding at high temperatures for increasing lengths of time should result in increasing periods of delay of the pre-division stage on returning the organism to optimum temperature. Have you any evidence of this?

E. Zeuthen. I think the evidence is that the holding of the organism at high temperatures for varying lengths of time does not influence the delay with which cleavage sets in after return to the optimum temperature. I agree that this makes hypothesis (*a*) less likely, and that therefore the possibility (*b*), or an even more complicated one, should be favoured.

A study of bacterial populations in which nuclear and cellular divisions are induced by means of temperature shifts

by

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A GROWING population of micro-organisms is a mixture of cells representing all phases of the division cycle. Studies on such a population, therefore, cannot lead to identification of the successive physiological and cytological states of a dividing cell, and very little can be learned about these states by observing individual cells undergoing division. Many problems concerning cell multiplication, which cannot be studied on single cells or on conventional cultures, might be solved by studying samples from a culture in which the cells were induced to divide more or less simultaneously. General considerations of this kind lead us to investigate possible means of producing the desirable 'phasing' or 'synchronization' of cell division in bacterial cultures.

Our choice of means was greatly influenced by the recent work of Scherbaum and Zeuthen (1954) and of Hotchkiss (1954), who have used temperature changes to obtain phasing of cultures with respect to certain physiological characters. Our approach is closer to that of Hotchkiss than to that of Zeuthen; and, since Zeuthen's studies are presented elsewhere in this symposium (Zeuthen and Scherbaum, 1954) we may at this point draw attention particularly to Hotchkiss's work (1954). In his experiments, periodic changes in rate of division, as well as in susceptibility to change into a streptomycin-resistant form, were induced by exposing the cultures to a temperature considerably below the normal, and optimal, growth temperature. Hotchkiss mainly used shifts between 37 and 25° C., and the same interval was adopted for our experiments with *Salmonella typhimurium*.

The strain we have employed has properties of particular value for the present investigation: firstly, young cultures of this organism consist of well-isolated cells and cell pairs with no tendency to clumping; secondly, the strain interacts with a certain bacteriophage thereby becoming changed in a characteristic and easily recognizable manner (Boyd, 1950; Lwoff, Kaplan and Ritz, 1954); and, finally, abundant growth can be obtained even in media composed of inorganic salts plus a simple carbon source such as glucose. The first of these properties has permitted

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the growth curves to be determined with satisfactory accuracy by direct colony counts; the second point, the interaction with phage, has been very helpful in that it has revealed periodic changes in susceptibility which, as will be seen later, appear to reflect nuclear doubling. The last property is potentially valuable because biochemical work, including tracer studies, can be carried out under well-controlled conditions.

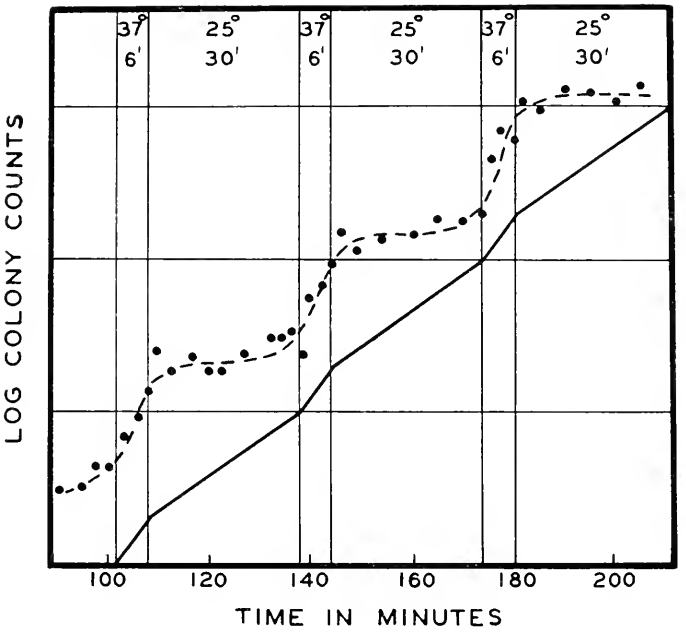
We shall first examine the type of growth curve which can be obtained by submitting the culture to cyclic changes of temperature. The technical details of the experiments will be described in a forthcoming publication (Lark and Maaløe, 1954); here a brief mention of the experimental conditions will suffice: acrated broth cultures, not containing more than 20 to 40 million organisms per ml., were used throughout; the temperature shifts were effected almost instantaneously by transferring the cultures to tubes kept at the desired temperature and adding hot or cold broth as required. No loss in viable counts has been observed following the sudden cooling or heating. One temperature cycle, consisting of changing the temperature from 25 to 37° C. and back to 25° C., involves a dilution of the culture by a factor 1·93, which is balanced quite accurately by the increase in colony count during the cycle. It is important to notice that colony counts can give very precise results, even when two large dilution steps are needed between sampling from the culture and spreading on agar plates, provided the experiment is designed in such a way that 300–600 colonies can be counted per sample. Under these conditions and with careful pipetting the standard deviation is about 5 per cent., which is close to the sampling variation to be expected on the assumption that the suspended bacteria are randomly distributed.

Figure 1*a* shows the step-wise rise in colony count regularly obtained after 2–3 conditioning cycles of temperature shift. In this experiment alternating periods of 30 minutes at 25° C. and 6 minutes at 37° C. were employed. The third, fourth and fifth cycles are represented by points corresponding to individual counts, and a continuous curve below the points shows the growth to be expected if the rate of division was always that characteristic of prolonged growth at the prevailing temperature. This lower, theoretical curve is based on control experiments yielding generation times of 45 to 50 minutes for growth at 25° C. and of 18 to 20 minutes for growth at 37° C. Two characteristics of experiments of this type should be emphasized: the virtual absence of cell division during about 25 minutes of each 25° C period, and the near equality of the experimental and the theoretical generation times. Together these observations show that a considerable degree of phasing or synchronization of cell division has been obtained, and that the temperature regimen employed probably has not impaired the vitality of the cells. It may be added that the steep portions of the experimental curves correspond to a division rate about twice that normally found at 37° C.

For convenience, all of the early experiments, including that of Figure 1*a*, were carried out with non-aerated cultures diluted beforehand to such an extent that plating on agar could be made without further dilution. This procedure had to be altered because the bacteriophage experiments as well as the cytological and biochemical studies we wanted to carry out on synchronized cultures require densities of at least 10 million organisms per ml. Systematic studies were therefore carried

out on sufficiently dense cultures, and Figure 1*b* shows the results obtained with a vigorously aerated culture containing about 30 million bacteria per ml.; under these conditions cycles of 28 minutes at 25° C. and 8 minutes at 37° C. gave the best results.

The type of experiment just presented was built up on the basis of preliminary investigations which will be described in more detail elsewhere (Lark and Maaløe, 1954). In this connexion we shall mention only two observations of particular significance. Firstly, it was found that the condition of the culture before temperature



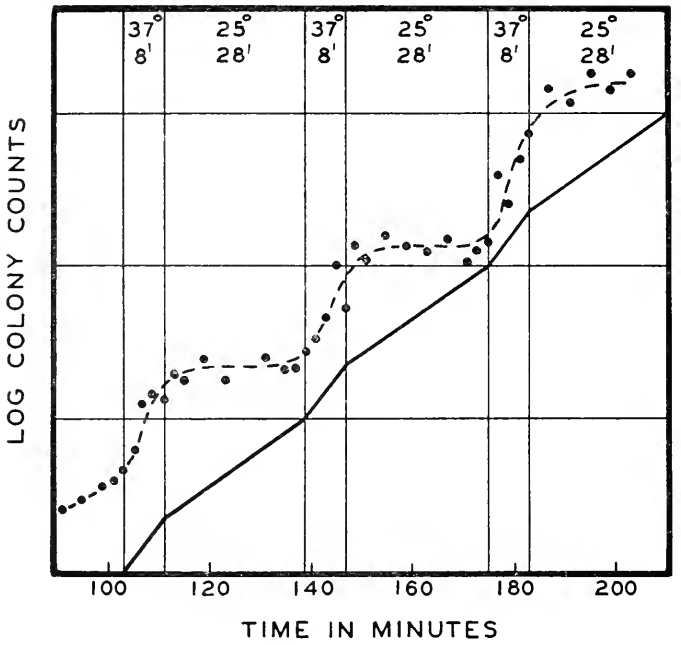
1*a*. *Experiment carried out with a non-aerated broth culture with about* 5000 *bacteria per ml. Points represent individual colony counts; the intervals between horizontal lines are* 0·3 *log units each, corresponding to a doubling of the colony counts. The continuous curve is theoretical; see text on page* 160.

Figures 1*a and* 1*b. Growth curves showing step-wise cell divisions.*

cycling is begun is important. The best results are obtained when a culture, grown to saturation at 37° C., is first diluted one hundred-fold and then aerated lightly for about 75 minutes before the first lowering of temperature takes place. After this preliminary period one ml. of the aerated culture contains 20 to 40 million organisms which have just entered the logarithmic phase of growth. Secondly, a peculiar observation was made concerning the behaviour of a culture when the temperature is raised to 37° C. after the first 25° C. period. If this period is extended to two or more hours, the rise in temperature is followed by a lag of some 20 minutes during which the rate of division is less than or equal to that of the preceding 25° C. period. After the first 20 minutes at 37° C. a sudden rise in colony count is registered before the normal 37° C. growth rate is established. This set of observations is illustrated

by the lower curve in Figure 3. It should be noted that the lag and the subsequent rise may easily be overlooked. As illustrated in Figure 3, the rise causes the linear portions of the growth curve to intersect near the zero-time axis, which means that the lag period will be registered only if a sufficiently large number of counts are made during the first 30 minutes after the temperature increase. The observations just described are, as we shall see later, of great significance for the experiments with bacteriophage and for the cytological studies to which we shall turn next.

The phage-sensitive strain of *Salmonella typhimurium* used for this investigation was obtained from Dr. Lwoff together with the two closely related bacteriophage strains



1*b*. *Experiment carried out with a vigorously aerated broth culture with about* 3×10^7 *bacteria per ml. For explanation see above.*

Figures 1*a and* 1*b. Growth curves showing step-wise cell divisions.*

A and *Ac*. The ways in which these phage strains interact with the host cells must be briefly reviewed. Infection of a sensitive bacterium with the *A*-phage leads to one of the two following results: *either*, the *A*-phage multiplies in the cell and this eventually lyses, *or* the infected cell survives and multiplies, and in that case it and its descendants have become immune both to the *A*- and to the *Ac*-phage (Lwoff, Kaplan and Ritz, 1954). The surviving cells give rise to pure cultures in which occasionally a cell lyses and liberates a large number of new *A*-particles; a culture of this kind is therefore called lysogenic. To account for the remarkable stability of such lysogenic cultures it must be assumed that the phage particle infecting the parental cell is completely integrated in the cell structure and that, from then on, it multiplies as part of the cell. In the integrated state, the phage particle is referred to as prophage,

and several independent lines of evidence suggest that this state arises if the infecting phage particle, or part of it, combines successfully with a bacterial nucleus (Murray, 1953; Lederberg, 1953; Jacob and Wollman, 1953; Appleyard, 1953). The prophage state, though very stable, is disrupted occasionally, with the result that unrestricted phage multiplication sets in, and, as mentioned above, the cell eventually lyses. The *Ac*-phage is a mutant derived from *A*, and it differs from the parental strain in that it nearly always causes lysis of the cell it infects; this mutant can be used, therefore, to eliminate non-lysogenic, sensitive bacteria from a mixed population of lysogenic and non-lysogenic cells (Lwoff, Kaplan and Ritz, 1954).

The system just described has been used to study the response of a synchronized culture to infection with the *A*-phage. Experiments of the following type were carried out at various times during synchronous division: a sample of the culture was diluted 1:10 into broth containing about 100 million *A*-particles per ml.; after exactly one minute a further 1:50 dilution was made to stop adsorption of phage particles onto bacteria and a sample of the infected and diluted culture was plated together with a large excess of *Ac*-phage. All these operations were carried out at 25° C. The *A*-phage adsorbs very rapidly onto bacteria; under the conditions just described, one minute is sufficient for about 40 per cent. of the cells to become infected. As there are about 100 phage particles per bacterium during this period the percentage of infected cells is independent of such fluctuations in bacterial concentration as may be encountered. On the agar plates the excess of *Ac*-phage eliminates the non-infected and still sensitive bacteria, while permitting the lysogenized and immune cells to form colonies.

Figure 2 shows the results obtained by calculating the frequency with which sensitive cells become lysogenic when exposed to phage *A* at various times during cyclic temperature changes. The striking feature of the curve is the rapid increase in frequency of lysogenization, by a factor of approximately two, which regularly occurs some minutes after raising the temperature to 37° C.; this is succeeded by a slow decrease which extends over most of the following 25° C. period. Later, when the results of the cytological observations have been presented, the abrupt rise in the lysogenization frequency will be correlated with important changes inside the cells. At this stage we can draw the provisional conclusion, however, that the rises and falls in the frequency curve reflect periodic changes in intracellular conditions which are important in deciding whether a cell is going to lyse or become lysogenic. This can be concluded because control experiments have shown that the percentage of infected cells is constant throughout the division cycle; in other words, the shape of the curve of Figure 2 is the same whether the frequency of lysogenization is expressed as fraction of the total cell count or as fraction of the infected cells only. In Figure 2 is drawn also a broken curve showing the increase in total colony count; it is important to note that the increase in frequency of lysogenization is much steeper than the simultaneously occurring increase in colony count which, as usual, begins some minutes before the temperature is raised.

The abrupt increase in frequency of lysogenization, which appears to be induced by raising the temperature to 37° C., is probably a more direct effect of the temperature change than is the increase in the rate of cell division. It therefore seemed likely that changes in the lysogenization frequency might be induced by a treatment which

would not at the same time affect the rate of cell division. To investigate this possibility, we returned to the simple system described earlier (p. 160). A culture was grown overnight at 25° C. from a very small inoculum; when a density of about 10 million organisms per ml. had been reached, the culture was aerated for 90 minutes before the temperature was raised to 37° C. During these 90 minutes and the following 60 minutes samples were withdrawn for determination of total colony counts and of lysogenization frequency as described above.

Figure 3 shows the results obtained just before and during the period after the temperature shift. For clearness of presentation, the points corresponding to the

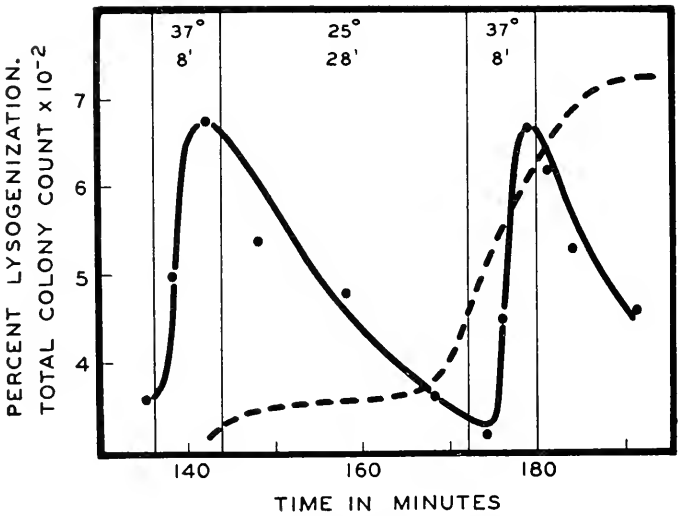


Figure 2. Frequency of lysogenization at various times during synchronous cell division. Points show individual frequency determinations based on total cell counts and counts of lysogenic colonies. The broken curve shows on an arithmetical scale the increase in total colony count, and thus corresponds to the growth curves in Figures 1a and 1b.

counts of lysogenic colonies have been plotted at a convenient distance above the points marking the total cell counts. The lower curve is an ordinary growth curve, representing viable counts, and it exhibits the characteristic lag, followed by an abrupt rise which already has been described (page 161). The upper curve, representing the lysogenic counts, and its relation to the growth curve may be interpreted as follows: before and during the first four to five minutes after raising the temperature to 37° C. the two curves are parallel, which indicates that the lysogenization frequency is constant; within the next three to four minutes the lysogenic counts double, and, since the division rate remains very low, this corresponds to approximately a doubling of the lysogenization frequency; then follows a period of 10 to 15 minutes during which the lysogenic counts are almost constant, and during which the lysogenization frequency gradually decreases as a consequence of cell division; finally, when normal growth has been established at 37° C., the lysogenic counts increase

at the same rate as the total cell counts and the lysogenization frequency assumes a constant value which is slightly higher than that characteristic of growth at 25° C. The insert in Figure 3 shows the changes in lysogenization frequency during the first 30 minutes; it should be added that the time after the change of temperature when the abrupt rise occurs is very constant from one experiment to another and that the rise is always close to two-fold.

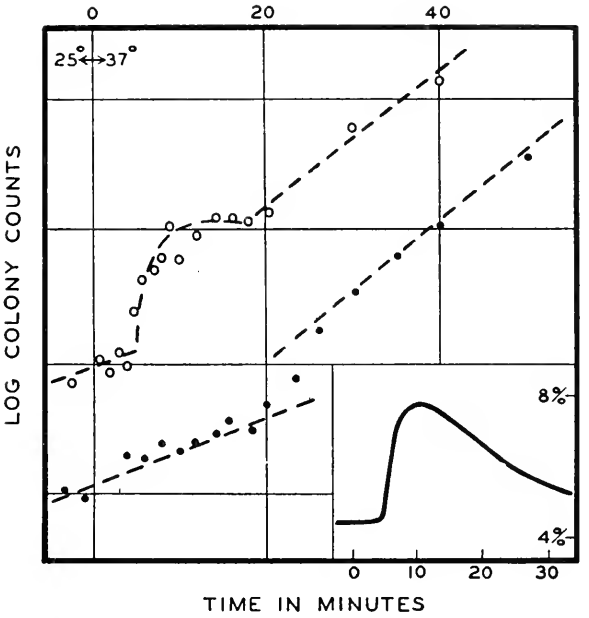


Figure 3. Growth and lysogenesis curves as influenced by a single temperature shift. Open circles (and upper curve) show counts of lysogenic colonies; points (and lower curve) represent total colony counts. The slopes of the early portions of the curves are determined by counts made during the 90 minutes preceding the temperature shift. As in Figures 1a and 1b the distance between horizontal lines corresponds to a doubling of cell counts. The insert in this figure illustrates the changes in the lysogenization frequency during the first 30 minutes of the 37° C. period, and should be compared with the continuous curve of Figure 2.

Before attempting to draw a comprehensive picture of the various effects of temperature changes on cell division and lysogenization we shall present the results of cytological studies made on samples taken during an experiment similar to the one presented in Figure 3. First a few words must be said about the fixing and staining techniques employed. Portions of culture were withdrawn and at once mixed with a solution of OsO_4 in buffered saline to give a final concentration of OsO_4 of 0 2 per cent.; after centrifugation the pellets were resuspended in small volumes of a 0 2 per cent. solution of OsO_4, and 20 minutes after the first mixing with OsO_4 loopfuls of the concentrated suspensions were spotted on dry agar surfaces. This somewhat

unorthodox procedure was adopted because we wanted to obtain fixation at a precise time relative to the time of the temperature rise and because it was important that no cytological changes should occur between sampling and the application of the fixing agent. After the process described above, impression prints were made on cover slips, and hydrolysis, staining with a thionine-thionyl chloride solution, and dehydration, were carried out as described by DeLamater (1952). The micrographs obtained in this manner do not compare in beauty with those made by expert cytologists using fixation in OsO_4 vapour; it is not known whether this is due to the fixation process we were forced to employ or to our lack of experience. The micrographs do show, however, that simultaneously with the rise in lysogenization frequency the number of stained spots, or 'nuclei', per bacterium doubles.

We shall continue to talk about these stained regions, in which the bacterial desoxyribosenucleic acid (DNA) is concentrated, as nuclei, remembering that we have no other way of defining a bacterial nucleus and that there are reasons to believe that it does not constitute as well defined an organelle as does the nucleus of cells of higher organisms (Birch-Andersen, Maaloe and Sjöstrand, 1953). The distribution of nuclei observed in the micrographs is as follows: while growing at 25° C., most cells have two and a few have four nuclei; during the period between 5 and 10 minutes after raising the temperature to 37° C., while the lysogenization frequency doubles, the fraction of cells containing four nuclei increases rapidly, so that by the time when no further increase in lysogenization frequency is observed the majority of the cells contain four nuclei and very few are found with only two. When the culture has continued growing for a couple of generations at 37° C. the distribution is again much like that observed before the temperature was raised; however, the proportion of cells with four nuclei is a little larger than it was when growth took place at 25° C.

Up to now we have presented some rather heterogeneous experiments, one after the other, without attempting to interpret the results. For practical reasons the experiments have been described in the order of increasing complexity, ending with the experiment in which cytological studies were included. Fortunately, these studies gave a clear-cut answer which makes it natural now to examine all our findings in the light of the cytological observations. These results may be summarized by stating that upon raising the temperature of a *Salmonella typhimurium* culture which has grown for some time at 25 to 37° C. nuclear division is induced in nearly all the cells.

We shall now discuss possible implications of this finding from different points of view:

(1) It is natural first to consider what mechanism may be responsible for the induction of nuclear division. The tentative explanation we have in mind is based on general ideas similar to those put forward by Hotchkiss, who writes as follows (Hotchkiss, 1954): '. . . the biochemical processes of cell growth and division would be disrupted in a systematic fashion by temporary exposure of a growing culture to a temperature well below that at which these systems had achieved a steady-state equilibrium. Certain of the enzyme systems should now be less able than others to transfer an amount of substrate equal to that supplied them, and there would then be a tendency for the metabolism to be selectively slowed or even temporarily halted at certain points.' Concerning the synthesis of DNA which must precede nuclear

division there is evidence from phage experiments which suggests that this process may be particularly sensitive to temperature changes. We have observed previously that phage synthesis, which to a very large extent means synthesis of DNA, has a temperature coefficient about twice as high as that of the rate of cell division (Bentzon, Maaloe and Rasch, 1952). DNA synthesis, in general, may therefore be selectively slowed down at low temperature; if so, the DNA precursors should accumulate when the temperature is lowered and reach a higher concentration in cells growing at 25° C. than in cells growing at 37° C. To account for the observation that nearly all the cells in a culture undergo nuclear division very soon after the temperature has been raised from 25 to 37° C. the following assumptions are made: firstly, that nuclear division is initiated when the precursor concentration reaches a critical level which depends on temperature; and secondly, that cells which recently have undergone nuclear division at 25° C. are left with a precursor concentration so high that a new nuclear division will be initiated if the temperature is raised to 37° C. This hypothesis is tentative, as already stated, but it should be possible to test it by means of biochemical studies using tracers to follow the assimilation from the medium of phosphorus and adenine, for instance.

(2) We shall now proceed to consider cell division. In this respect, the most striking observation made on our *Salmonella typhimurium* strain is that there seems to be an interval of more than half a generation time between nuclear and cellular division. This is most apparent in the experiments in which only one temperature change is involved; here nuclear division occurs in nearly all cells right after the increase in temperature, and the 'burst' of cell divisions corresponding to the nuclear division occurs 12 to 15 minutes later. It looks as if nuclear division actually blocks cell division for a considerable time. The experiments in which synchronous cell division was obtained also suggest that the cells do not divide until a long time after nuclear division has taken place; we shall return to this type of experiment later when discussing the process of lysogenization.

Our observations on cell division in *Salmonella typhimurium* have been made under rather artificial growth conditions as far as temperature is concerned. However, we assume that the time relation between nuclear and cellular division, which we have observed, also applies to cells growing at a constant temperature. In support of this assumption, we may cite the observation, made earlier, that the overall division rate in a synchronized culture is almost as high as might be expected on the basis of the division rates at 25 and 37° C. and the times spent at the two temperatures. The regimen employed to obtain synchronization, therefore, seems not to impair the growth and division processes appreciably; this, we believe, would not be the case if the temperature shifts interfered seriously with the natural sequence of events during the division cycle.

As a basis for later discussion, we may point to the striking difference between the division cycle in cells of higher organisms and that observed in *Salmonella typhimurium*. Most animal and plant cells are uninuclear, which means that, normally, cell division must follow right after nuclear division; in the multinuclear bacterial cells which we have studied, the opposite seems to be normal.

(3) Finally, we want to consider the process of lysogenization and its possible relation to nuclear division. It was mentioned earlier that the establishment of the

prophage state seems to involve an interaction between phage material and a bacterial nucleus (Murray, 1953; Lederberg, 1953; Jacob and Wollman, 1953; Appleyard, 1953). If only one phage particle enters the cell the probability of such an interaction occurring before unrestricted phage multiplication begins is rather low; it has been shown, however, that this probability, i.e. the lysogenization frequency, can be increased from 15 to 20 per cent. up to about 80 per cent. by increasing the number of phage particles per bacterium (Boyd, 1950; Lwoff, Kaplan and Ritz, 1954). Our experiments show that an increase in lysogenization frequency can be caused also by increasing the number of nuclei per cell. From these observations it appears probable that the lysogenization frequency is determined by the number of encounters between the entering phage particle, or particles, and the nuclei of the cell, which occur within a limited time after infecting the cell. In our experiments the conditions of infection were such that 80 to 90 per cent. of the infected bacteria received one phage particle only; and in such experiments nuclear doubling, throughout a culture, seems to cause a doubling of the lysogenization frequency.

What remains to be interpreted is the gradual decrease in lysogenization frequency which is observed in the synchronized cultures during the periods while little or no cell division takes place (see Figure 2). We believe that just after the 37° C. period when the lysogenization frequency has reached its maximum, all cells have doubled their nuclei; during the following 20 to 25 minutes the cells prepare for division, presumably by developing some kind of internal separation. Gradually all the cells would thus in a functional sense become double cells. In this way we would pass from a situation in which nearly all cells have, say, four nuclei within one functional unit, to a situation in which nearly all cells consist of two functional units, each with two nuclei. In our lysogenization experiments each infected cell unit, including the hypothetical double cells, adsorbed one phage particle only, and in the case of a double cell only one compartment of the cell would therefore be infected. It follows, from what was said above, that the postulated segregation process by which the number of nuclei per functional unit is reduced to one-half should be accompanied by a similar reduction in lysogenization frequency. This analysis of the lysogenization curve of Figure 2 brings us back to the idea that nuclear division is not immediately followed by cell division, which seems to occur only after a long period of preparation.

Acknowledgements. We wish to express our thanks to Dr. Lwoff for making available to us the bacterial strains and the phage strains used, and for pointing out some of their great advantages. We also wish to thank Dr. Kauffmann for generous gifts of specific antisera. We are indebted to Dr. Hotchkiss and Dr. Zeuthen for detailed and valuable information about their work prior to its publication. We are grateful to Mr. O. Rostock for expert assistance in the execution of most of the experiments.

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Discussion

Chairman: J. F. Danielli

K. E. Cooper. Does the resistance of these cultures to antibiotics change in their different phases? We have found a critical time in the development of staphylococci on solid media at about four generations after pouring inoculated plates to test antibiotic action (1952, *J. gen. Microbiol.* **7**, 1–17). A temporary increase in resistance occurs, and may be related to the time required to form some cellular substance after the act of inoculation.

O. Maaløe. Unfortunately we have no information as yet as to whether bacteria are particularly susceptible to antibiotics at certain points of the division cycle.

C. Darlington. With regard to the suggested high temperature coefficient of DNA synthesis, in those flowering plants in which mitosis takes place at freezing-point, a starvation of DNA often occurs at specific points in the chromosomes, the segments of heterochromatin, when the temperature is applied for some time before metaphase.

M. M. Swann. Unlike what happens in plants and animals, there is a considerable separation in your organisms between nuclear division and cell division. However, the partition is laid down earlier, and the 'cell division' appears to be a separation of what are already functionally distinct units. Perhaps then we may regard cytoplasmic division even in this case as essentially following immediately after nuclear division.

K. E. Cooper. Have you tried cell wall stains? Many bacteria have transverse septa which divide them into multicellular organisms.

L. Rinaldini. Has any electron-microscopy been done on these cells during the decline in lysogenesis, to see if the septum develops gradually in each cell or if it appears abruptly and the gradual decline is obtained by more and more cells dividing?

O. Maaløe. Studies by means of sectioning and electron-microscopy are in progress. At the moment we can say only that there is no indication of a morphologically well-defined septum being formed before cell division begins.

E. Ambrose. Is there any change in the sensitivity of the bacterial nuclei to phage at various stages of the division cycle? With larger cells the nuclear membrane disappears during metaphase. It seems likely that the phage nucleoprotein, which is of high molecular weight, could combine more easily with the nuclear material in the absence of a membrane.

O. Maaløe. The available data seem to indicate that there is no particularly sensitive period during the division cycle. With a high multiplicity of infection as many as 80 per cent. of the bacteria become lysogenic. It is not likely that within the short time during which the decision for or against lysogenization is made, the majority of the cells would pass into any specified phase of the division cycle. Also, if a particularly sensitive phase existed, we might expect the peak in the frequency curve to rise by more than a factor of two. As to the second point, there is some evidence that in the bacteria we have studied there is no well-defined nuclear membrane.

E. N. Willmer. I should like to call attention to some observations, on tissue cultures of chick fibroblasts, which seem to be somewhat parallel to those reported by Dr. Maaløe. These cells, when grown in flasks in a plasma medium, cease to show cell divisions in the outgrowth after about sixty hours; but, if they are then treated with embryo juice for about an hour, they can be caused to divide again for a limited time; such divisions start after about ten hours and cease after about twenty-four hours. Repetition of the dose of embryo juice before the cells start to divide is without effect, but if the second dose is delayed until the cells are dividing, then a second crop of divisions results in due course: thus, the activating agents, possibly with nucleoproteins among them, can only gain limited access to the cells and the latter can become 'saturated' until the situation is changed by the occurrence of the division process. (Jacoby, F., Trowell, O. H. and Willmer, E. N. (1937). *J. exp. Biol.* **14**, 255.)

Secondly when chick cultures are cooled to a temperature below 10° C. divisions temporarily cease, but when the cultures are returned to 37° C. there is a compensatory excess of cell divisions. (Spear, F. G. (1928). *Arch. exp. Zellforsch.* **7**, 484.)

By combining these two sets of observations a method for obtaining more nearly synchronous mitoses in chick fibroblasts would appear to emerge as a possibility.

O. Maaløe. I am very pleased that a point like this has been raised, because I feel that in our paper it is not emphasized strongly enough that temperature shift is but one means out of a great many which may be tested for their suitability to produce synchronous behaviour in cell populations.

Environmental and genetic control of differentiation in Neurospora

by

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As is well known, it has been possible in recent years to obtain very precise information about the biochemistry and physiology of growth and its genetic control in certain fungi and bacteria by means of a very ingenious technique. The 'wild type' strain of the organism is grown on a synthetic 'minimal medium'. Mutants are produced which cannot grow on the minimal substrate but only on a 'complete medium' supplemented with various diffusible growth factors. The mutants lack the capacity to carry out a certain enzymatically controlled metabolic step, necessary for normal growth, and this step can be identified by adding only one growth factor at a time to the minimal medium. Hence the mutation serves the purpose of a very specific enzyme inhibitor, and the advantages of the method have been amply demonstrated in studies of the biosynthesis of various amino-acids and vitamins.

This method has almost exclusively been applied to the study of growth, whereas very little work along similar lines has been done on morphogenesis in these organisms. It would be interesting, however, to use the technique which has been so successful for the study of growth to a study of problems of differentiation. This would be possible, (*a*) if one or several well-defined steps in morphogenesis could be controlled on a synthetic medium, (*b*) if mutants could then be produced in which normal differentiation was either blocked or modified on the minimal medium, and finally (*c*) if normal differentiation could be induced again in these mutants by supplementing the minimal medium with suitable precursors.

The first difficulty is obviously that of selecting a suitable system of differentiation which can be properly controlled on a synthetic minimal medium. The selection of such a system in fungi and other micro-organisms is not too easy, because the morphogenesis of micro-organisms is far less well defined and far less advanced than that of higher plants and animals. Let us take as an example the mould *Neurospora*, a well-recognized tool for genetical experiments since the earlier work of Dodge and Lindegren and the more recent work of Beadle, Tatum and their group. It will be seen from Figure 1 that *Neurospora* during development does three different things: (1) it grows; (2) it forms conidia; and (3) it forms, on the haploid mycelium, female

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sex organs, the so-called protoperithecia, which after fertilization develop into perithecia.

It might *a priori* be considered possible to select growth as such for our purpose, because the growth type of *Neurospora* is easily modified by changes of substrate (Tatum, Barratt and Cutter, 1949) and because a number of morphological mutants of widely different growth type are available (Barratt and Garnjobst, 1949; M. and H. K. Mitchell, 1952; and others). The study of the biochemical genetics of some of these mutants is now in progress (Mitchell, Mitchell and Tissieres, 1953) but it is

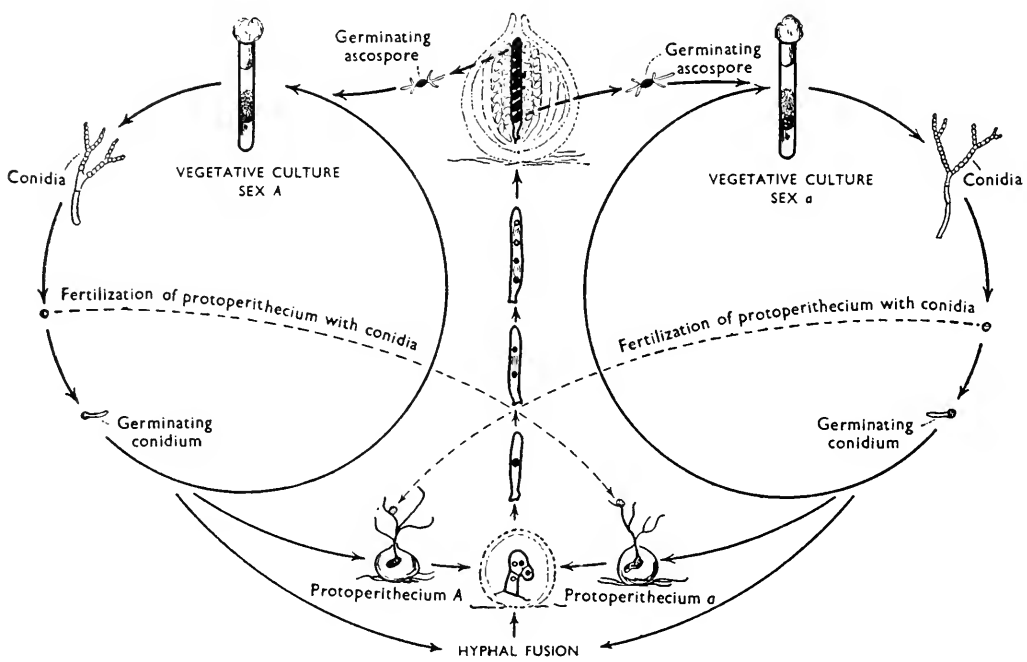

Figure 1. *Diagram of the life cycle of* Neurospora. (*From Beadle in* Amer. Scientist, 1947.)

still too early to form an opinion of the possibilities of this system for studying problems of differentiation. As is well known a similar system has been studied intensively in yeast by Ephrussi and his group. The implications of their results on general problems of morphogenesis has recently been summarized by Ephrussi (1953).

The second differentiation process in *Neurospora* which might be selected for a study of this kind would be the formation of conidia. This also is strongly influenced by the composition of the substrate, and a number of mutants are available which either form abnormal conidia or fail to form any at all. It is also known that differentiation of conidia is associated with the production of yellow pigments identified as carotenoids (Haxo, 1949; Sheng and Sheng, 1952). However, everybody familiar with work on *Neurospora* will know that the formation of conidia is a very complicated process involving the production of both macroconidia and uninucleate microconidia,

and although it might ultimately be possible to work out the biochemical genetics of this process in some detail, the system is certainly not an attractive one.

This leaves the formation of protoperithecia as the third alternative which may be suitable for our problem. The formation of these bodies on haploid mycelia is not a very common phenomenon in the fungi, and it is most fortunate that *Neurospora* belongs to the group of Pyrenomycetes which have them. They had already been reported in the classical paper by Shear and Dodge (1927). However, their function as female sex organs was first described by Dodge and Swift (1933) and by Dodge (1935). Their development was later studied in some detail by Backus (1939). Despite these investigations we are far from having sufficient information about their detailed structure and development.

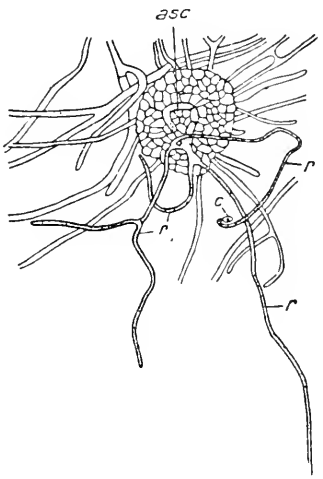

Figure 2. The structure of a protoperithecium of Neurospora*;* asc*: ascogonium;* r*: receptive hyphae (trichogyne);* c*: conidium which fertilizes the receptive hyphae. (From Backus,* 1939.)

When *Neurospora* is grown under suitable conditions to be defined later, the protoperithecia begin to develop in abundance after 3–4 days, and they can then be fertilized. They are shown in Plate I*a*; Figure 2 shows some details of their structure based upon Backus's paper. We have made some preliminary investigations, trying to follow the first steps in their differentiation. First, certain vegetative hyphae begin to curve and form small spirals. These hyphae stain deeply with cotton blue. Next, these spirals seem to attract hyphae from the surrounding mycelium, and the primordium (the ascogonium) becomes surrounded by dense balls of deeply staining hyphae which after 3–4 days are easily recognizable under a low-power microscope. The differentiation of these organs is thus a very well-defined and conspicuous process which takes place within very few days, and which among the three possible systems (growth type, formation of conidia, or protoperithecium formation) seems to be by far the best suited for our purpose.

Having thus selected the differentiation of the female sex organs as our morphogenetic system, the next problem is to control this differentiation on a synthetic medium. The minimal medium used in standard work on *Neurospora*, the so-called 'Fries-minimal medium', does not allow protoperithecia to develop, and consequently sexual reproduction does not take place on this substrate (Table I). Until recently *Neurospora* was grown on cornmeal agar for sexual reproduction, and this of course is not a synthetic medium. In 1947 Westergaard and H. K. Mitchell worked out a synthetic medium which allows the formation of perithecia and abundant sexual reproduction to take place. This was the first step in the development of a system allowing a study of the biochemical genetics of protoperithecial differentiation. This medium, which we call the 'P-minimal medium' (perithecia-promoting medium), differs from the standard Fries-minimal medium in containing no ammonium ions; the pH is adjusted to 6·5 as compared to 5 5 in the Fries-medium

TABLE I

Composition of different media per litre of H_2O.

Substrate*	Ammonium-tartrate	Potassium-tartrate	KNO_3	NH_4NO_3	pH
Fries	5 g.			1 g.	5·6
P-minimal			1 g.		6·5
Horowitz		6 38 g.	3 g.		5·6

* All substrates have in addition: KH_2PO_4 1 g., $MgSO_4$ 0·5 g., $CaCl_2$ 0·1 g., NaCl 0·1 g., sucrose 15 g., biotin, and trace elements. The low-sulphur media have 0·0079 g. $MgSO_4$ instead of 0·5 g. and in addition 0·4 g. $MgCl_2$.

(Table I). In the same paper it was shown that the development of perithecia depends also upon the carbon/nitrogen ratio, upon pH, and upon temperature. The optimal temperature for perithecium formation is 25° C., whereas no differentiation at all takes place at 35° C.

The work of Westergaard and Mitchell was extended by Dr. Herbert Hirsch who worked in our laboratory for two years. His results have recently been published (Hirsch, 1954). He was able to confirm the preliminary observations by Westergaard and Mitchell on perithecium formation, and he extended them to protoperithecium formation; he made the discovery that the latter system is a reversible one. If slants are incubated for 7–14 days at 35° C. on P-minimal medium (i.e., under conditions where no protoperithecia develop) and then transferred to 25° C., protoperithecia develop after a few days. If, on the other hand, protoperithecia are allowed to develop at 25° C. and the slants are then transferred to 35° C., the protoperithecia can no longer be fertilized. We believe that this reversibility will be of great use in future studies.

Hirsch also made the first studies on the biochemical mechanism involved in protoperithecium formation. Starting from the observations that mycelia in which protoperithecia are formed in abundance turn brown or black, that both mature

protoperithecia, perithecia and ascospores contain a black pigment, and that strains growing under conditions where no protoperithecia develop never turn brown or black, it was assumed that these pigments were melanins and that a causal relationship exists between melanin metabolism and protoperithecium formation. The black pigments associated with protoperithecium formation were identified as melanins by various chemical reactions, by studying their absorption spectra, and by the presence of a tyrosinase. No melanins could be demonstrated in mycelia on which protoperithecia were not found.

This observation of course made it important to study tyrosinase activity in greater detail in strains growing under different conditions, favouring or suppressing protoperithecium formation, as it is well known that tyrosinase converts tyrosine or other

TABLE II

Tyrosinase activity of ground suspensions of Neurospora crassa. *Strain W 2/49 A grown for 7 days on filter-paper on*: (1) *P-minimal medium at* 25° *C.*; (2) *P-minimal medium plus* 3,000 *mg. of amino-N (hydrol. casein A) per litre at* 25° *C.*; (3) *P-minimal medium at* 35° *C. The figures given are net increases (zero time readings deducted) in optical density, multiplied by* 100.

Time (minutes)	25° C. mycelium	25° C. AA-mycelium	35° C. mycelium
0	0	0	0
15	2·5	0·6	0·2
30	6·1	1·1	0
45	12·4	2·3	0
60	19·2	3·7	0·4
Dry weight of mycelium (mg. per 0·5 ml. of suspension used)	7·6	9·0	8·2

substrates into melanins via dopa, dopachrome and a number of other intermediates (see Lerner, 1953).

Hirsch showed that no tyrosinase activity could be demonstrated in strains grown at 35° C., whereas tyrosinase activity was present in mycelia grown at 25° C. (Table II). As shown in Figure 3, tyrosinase activity on P-minimal medium at 25° C. started on the third day, reached its maximum on the fourth day, and then went down again. It will be noticed from the figure that the highest tyrosinase activity was found in the period when protoperithecium formation was most intense.

Table II also includes an experiment in which *Neurospora* was grown on P-minimal supplemented with 3,000 mg./litre amino-N (the AA-mycelium). Very little tyrosinase activity was found in mycelium grown on this substrate in 7-day old cultures, and the red pigment did not turn black (melanic), as did the pigment in culture grown

on P-minimal medium. After 2 weeks, however, when tyrosinase activity in mycelia grown on P-minimal medium went down, the activity was very much increased on the AA-substrate, and now the pigments turned black during the enzymatic test. No protoperithecia developed on the AA-mycelium with 3,000 mg./l., nor was melanin formed during actual growth. At a lower concentration (1,500 mg./l.), protoperithecia were formed after 2 weeks, but they could not be fertilized.

Hirsch made further experiments to test the hypothesis that there is a connexion between tyrosinase and differentiation. A number of tyrosinase inhibitors (*p*-aminobenzoic acid, sodium-thioglycolic acid, cystein and phenylthiourea) were added to P-minimal medium. They were all able to suppress protoperithecium formation more or less completely, whereas other enzyme inhibitors without specific affinity towards

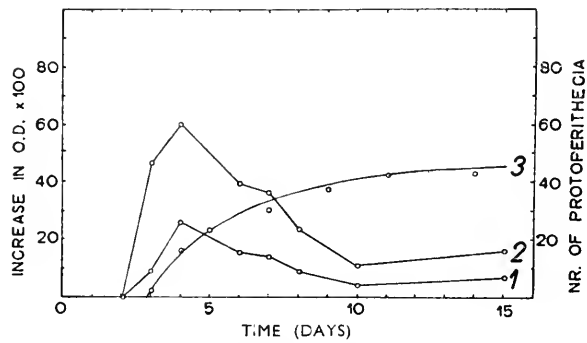

Figure 3. Tyrosinase activity and protoperithecium formation as a function of time in Neurospora crassa *strain W 2/49 A, grown on P-minimal medium at 25° C. Curve 1: tyrosinase activity of ground suspensions; net increase in optical density × 100 in 30 minutes. Curve 2: ditto; net increase in optical density × 100 in 60 minutes. Curve 3: number of protoperithecia. (Hirsch, 1954.)*

the tyrosinase system (e.g. streptomycin and others) did not interfere with the differentiation process.

It might be mentioned at this point that the present differentiating system has certain advantages over the growth-system studied by other workers: it is possible to study the metabolism of mycelia in which differentiation is blocked. The same, however, cannot be done with mycelia in which growth is blocked, because the alternative to growth is lethality. Thus, from studying the metabolism of mycelia in which differentiation was blocked by various environmental factors, it has been possible to get information about the biochemistry of differentiation even without bringing mutant strains into the picture.

However, the relationship between tyrosinase activity, melanin formation and development of female sex organs is far more complicated than might appear from the data hitherto presented. In recent years tyrosinase activity in *Neurospora* has been studied by Fox and Gray (1950), and especially by Horowitz and his co-workers (Horowitz and Shen, 1952; Horowitz and Fling, 1953; see also Horowitz, 1951).

None of these authors, however, connected their observations with the differentiation of the sex organs. Fox and Gray claimed that there is a difference in tyrosinase activity between the two mating types (+ and −) of *Neurospora*, an observation which was not confirmed either by Horowitz or by Hirsch. Horowitz and Shen also found, as did Hirsch, that tyrosinase activity in *Neurospora* depends upon the temperature. Moreover, they made the interesting discovery that, under the conditions and with the strain which they used, an inhibitor was present which suppressed tyrosinase activity. Horowitz used a modified, liquid Fries-medium ('Horowitz-medium'). Its composition is shown in Table I. They showed that this inhibitor disappeared if the mycelium was dialysed. Its presence in non-dialysed mycelium was demonstrated by adding fresh mycelium to a tyrosinase preparation, after which procedure the tyrosinase activity was inhibited. They also found that the tyrosinase inhibitor disappeared when *Neurospora* was grown on a medium poor in sulphur.

This brings the possible connexion between the tyrosinase inhibitor and protoperithecium formation into the picture. Hirsch found that the inhibitor was present only in mycelia grown in liquid cultures; it was present whether Horowitz or P-minimal medium was used. No protoperithecia were ever formed in liquid cultures. If, however, the mycelia were grown on filter paper in liquid cultures, tyrosinase activity was present in non-dialysed mycelia grown on Horowitz as well as on P-minimal medium, and protoperithecia were formed in both cases. On the other hand, tyrosinase activity was found in non-dialysed mycelia grown on Horowitz low-sulphur medium, but hardly any protoperithecia developed on this substrate. Finally: if the standard P-minimal medium was substituted with a P-minimal low-sulphur medium, protoperithecium formation was almost as good as on standard P-minimal. Evidently until more is known about the nature of the suppressor present in the mycelia grown in liquid media, its pH-dependence, its interaction with the constituents of the different media and variation between different strains, we shall have to leave this problem open.

Horowitz and Fling later made a more thorough study of the sensitivity of *Neurospora* tyrosinase to temperature. They found another strain of *Neurospora* containing a temperature-stable tyrosinase. Unfortunately nothing is said about sexuality in this strain. The tyrosinase from the temperature-unstable strains was found to have a half-life of 3–4 minutes at 59° C., whereas the enzyme from the stable strains had a half-life of at least 30 min. at the same temperature. The difference between the two strains was controlled by a pair of allelic genes designated T^S/T^L. The absence of tyrosinase activity at 35° C. in temperature-sensitive strains was not due to a suppressor (Horowitz and Fling, l.c.; Hirsch, l.c.).

We have so far been dealing with only one part of the problem, viz. the environmental control of differentiation in *Neurospora*. As a working hypothesis we want to suggest that there is a causal relationship between tyrosinase activity and melanin formation on one hand and differentiation of female sex organs on the other hand. It also seems as if normal differentiation can be blocked at high temperature because one of the enzymes involved in melanin metabolism is temperature-sensitive, the temperature-dependence of the enzyme being under genic control. Although the evidence is far from conclusive, there may also be a relationship between the formation of a tyrosinase inhibitor on certain substrates and the scarcity or absence of

protoperithecia on the same substrates. Hence, genes and environment together determine a series of different states, showing different morphological and biochemical characteristics.

Turning now to the second, or genetical part of the problem, it is evident that the control of differentiation on a synthetic medium has been worked out sufficiently to allow a study of mutants in which protoperithecial development is either blocked or altered on the P-minimal medium. It has not been necessary to produce such mutants experimentally. It is a well-known fact that most *Neurospora* strains become more or less sterile when they are propagated vegetatively over a long period. From such old strains we have isolated a number of mutants in single-spore cultures which show various degrees of sexual sterility. Some of these mutants never form protoperithecia on P-minimal medium, and they are completely female-sterile (Plate I*b*). Fortunately, however, most of them are male-fertile so that they can be studied genetically. Other mutants have abnormal-looking protoperithecia which cannot be fertilized, and we have still other mutants which do develop protoperithecia and after fertilization also normal-looking perithecia, which, however, never contain ascospores. The strains which do not produce protoperithecia do not turn dark, confirming the evidence from the first part of the investigation, that there is a relationship between the formation of protoperithecia and of melanins. We have tested three of these mutants for tyrosinase activity and have found none.

On the other hand we have a very interesting group of mutants which produce a very great number of small protoperithecia; these strains turn completely black in a very short time and they are completely female-sterile (Plate I*c*). Apparently both absence of melanin formation and excessive melanin formation interfere with sexual differentiation. Although the formal genetics of the various sterile mutants has not yet been worked out, there is some evidence both from heterokaryons and from other experiments that we are dealing with different, non-allelic mutants.

If the standard biochemical genetical technique were to be applied to these mutants it would mean feeding the different mutants with different intermediates (precursors) in melanin metabolism and studying the reaction of the mycelia on the supplemented media. (It is a noteworthy fact that none of the sterile mutants become fertile on the standard 'complete' media, which are supplemented with vitamins, casein-hydrolysate, yeast and malt extract). Unfortunately a number of difficulties arise here. For one thing it is unlikely that anything would come out of feeding the melanins directly to the mutants since it is unlikely that such high-molecular compounds would penetrate the mycelium. For the same reason it seems unlikely that adding tyrosinase itself to the substrate would have any effect. Unfortunately the various low-molecular intermediates in melanin synthesis have not been available to us (see Lerner, 1953). We have therefore tried a short-cut. Tyrosine + tyrosinase prepared from cultivated mushrooms (*Psalliota*) were added to sterile mutants which were grown for 3 days on P-minimal medium, in the hope that some of the intermediates formed during melanin synthesis might be picked up by the mycelium. Somewhat to our surprise one of the mutants (913/83) gave what might be called a promising reaction to this crude treatment, forming, where substrate + enzyme was added, protoperithecium-like structures (Plate I*d*). The induction of these structures, although they were far from being normal protoperithecia, certainly suggests that

tyrosinase-melanin intermediates have a morphogenetic capacity in the protoperithecial system. These observations, together with the evidence already presented, suggest that our working hypothesis of a connexion between differentiation of protoperithecia and synthesis of melanin in *Neurospora* is a sound one. To these observations should be added a rather interesting preliminary observation on a sterile mutant (93/54) which never forms protoperithecia on the standard P-minimal medium, whereas it does form many protoperithecia on a low-sulphur P-minimal medium. This suggests that in this mutant the tyrosinase inhibitor of Horowitz and Shen, which normally is inactive on the standard P-minimal medium, is involved.

It is quite obvious that our technique for feeding precursors in this system is much too crude. The various assumed intermediates in melanin metabolism will have to be synthesized and fed individually to the mutants before we can get much further. What we want to present here is a morphogenetic system, namely the formation of protoperithecia, which offers promising opportunities for studying the genetics and biochemistry of morphogenesis on a synthetic substrate; and we hope to have presented convincing evidence for our working hypothesis that at least one of the keys to the biochemistry of this problem is tyrosinase activity and melanin synthesis.

Before finishing this presentation it may be worth while to discuss some of the more general aspects of this work. As pointed out already by Dr. Horowitz, melanin problems present one of the few fields where biochemistry, embryology, and genetics meet, and, as Dr. Horowitz has also pointed out, with respect to tyrosinase activity *Neurospora* behaves like the Siamese cat or the Himalayan rabbit. To this we want to add that in *Neurospora* this enzyme system is involved in sexuality. We now also have *Neurospora* mutants lacking tyrosinase and unable to form melanin, thus behaving like many albino animals. In other mutants melanin formation is inhibited, just as we have albinos in animals due to epistatic suppressors. We have certain mutant strains in *Neurospora* where the melanin seems to agglutinate, bringing the dilution gene (D-gene) in rabbits to mind. This investigation might also be discussed in relation to the older work of Goldschmidt on the effect of temperature upon the differentiation of the wing pattern in butterflies (see Goldschmidt, 1938). It should also be remembered that we are dealing with the very first system from which biochemical genetics emerged: the work of Garrod on inborn errors of metabolism in man (Garrod 1923; Haldane 1954). Although we may not expect to find *Neurospora* strains with alcaptonuria, hydroxyphenyluria, etc., we may nevertheless have a system which can be of importance for studying some of the biochemical blocks which in man are so often connected with mental disorders.

A second aspect of this investigation is its relationship to the possible occurrence of sex hormones in fungi, a subject recently reviewed by Raper (1952). In *Neurospora* the earlier claims on the occurrence of diffusible sex hormones (Moreau and Moruzzi, 1931) have been refuted (Aronescu, 1933; Hirsch, 1954). As far as the higher fungi are concerned, it seems a good idea to look for the intermediates in melanin synthesis in future work. Also the work of Moewus on sexuality in *Chlamydomonas* which, if taken at its face value, represents a system far better worked out in detail than any other, should be kept in mind. As will be remembered Moewus claims that two quite unrelated groups of chemicals, carotenoids and anthocyanins, play a role in sex

differentiation in *Chlamydomonas* (see Moewus, 1950). We may take this as a warning against restricting the biochemical part of this work to the melanins only.

The third implication, and still of course a purely speculative one, might also be mentioned here. Melanins are of widespread occurrence among the higher fungi, both in the Ascomycetes and Basidiomycetes; mushrooms like *Psalliota* and *Lactarius* are well-known sources of tyrosinase. The pigments seem always to be associated with reproduction. This brings into the picture the so-called *Fungi imperfecti*—the fungi which only reproduce asexually, the sexual (perfect) stage being unknown. It would be interesting to compare tyrosinase activity in some of the asexual species with that of related sexual species (e.g. *Aspergillus niger* with *A. nidulans*, *Penicillium chrysogenum* with some of the sexual species) and study the reaction of the asexual species to melanin precursors. It would have considerable theoretical as well as practical implications if it should prove possible to induce sexuality in some of the asexual fungi, among which we find some of the most important industrial species.

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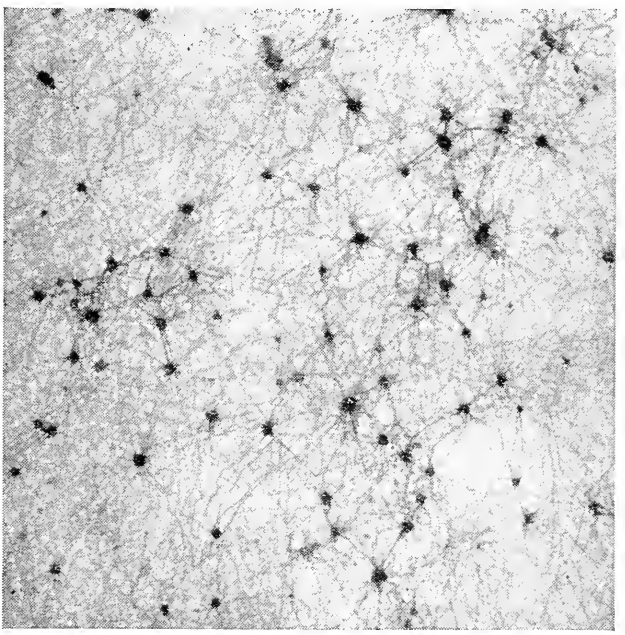

Plate 1 (*a*)

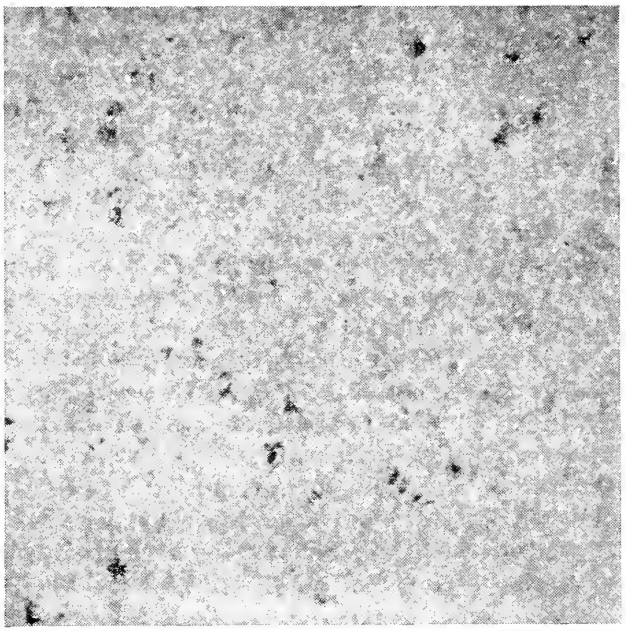

Plate 1 (*b*)

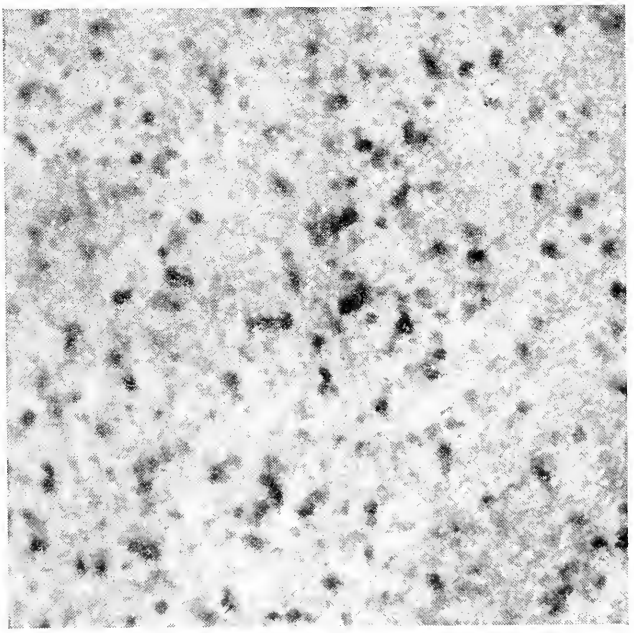

Plate 1 (*c*)

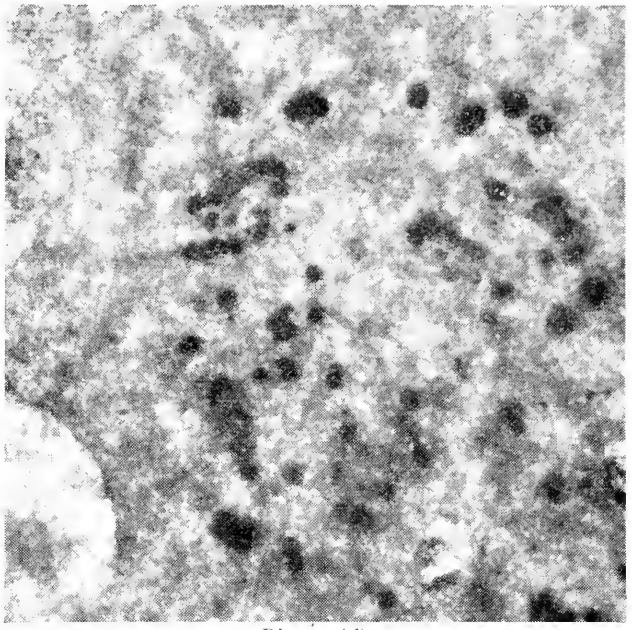

Plate 1 (*d*)

Plate I. (*a*) *protoperithecia in normal* Neurospora grown on *P-minimal;* (*b*) *sterile mutant grown on the same substrate, no protoperithecia are formed;* (*c*) *mutant with abnormal protoperithecia, associated with excessive melanin production;* (*d*) *3-day-old culture of a sterile mutant to which tyrosine + tyrosinase has been added.* (*Photos by A. Oye, linear magnification* × 20.)

Discussion

Chairman: H. V. Brøndsted

E. N. Willmer. What do you consider to be the relationship between the formation of melanin and that of carotenoids? In the chromatophores of vertebrates, which are derived from neural crest tissue and are thus among the earliest cells to begin differentiation in the body, the formation either of melanin or of carotenoids is of frequent occurrence. Occasionally both products occur together. The other tissues in the body where carotenoids abound are of course those in the reproductive system and in the adrenal cortex. In the latter case it is interesting to consider whether there may be some connexion between the carotenoid pigmentation of the cortex and the tyrosine-adrenaline metabolism of the medulla. It is well known that adrenal cortical disease often leads to abnormalities of melanin formation.

M. Westergaard. Wild type *Neurospora* has both carotenoids and melanins. We may consider the conidia, which have the carotenoids, as part of the male sex system; just as the protoperithecia, which have the melanins, represent the female sex system. Mutation in one system does not affect the other, i.e. strains with no conidia may have perfectly normal protoperithecia and vice versa. Unfortunately the male sex in *Neurospora* is rather degenerate, because fragments of mycelia can also fertilize the protoperithecia. Probably the biochemistry of the male sex should be studied on a species where it is better differentiated.

The control of cell division

by

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INTRODUCTION: DIVISION WITH GROWTH

CELLS maintain a roughly constant size*, from which it has been argued that division must be dependent in some way on growth. Lewis (1948), for instance, says that if this were not so, cells might be expected to get smaller and smaller or larger and larger. It must be admitted, however, that in the few cases where serious attempts have been made to measure the size of cells, it seems that they do sometimes get smaller and smaller or larger and larger. In the growth of protozoan cultures for instance, cell size varies within wide limits at different stages. Comparable variations are to be found in the growth of bacteria and yeast in cultures. Even in the case of somatic cells, where the difficulties of measurement are considerably greater, there are scattered references to variations in cell size under different conditions.

Nevertheless, to a first approximation, cell size does remain constant. It follows that size plotted against time for a given line of cells must give a relationship of the general type shown in Figure 1*a*. The precise form of this relationship has in fact never been settled, because of the extreme difficulty of determining the size of a single cell. There is some evidence from measurements made on Protozoa that the main growth in volume, and perhaps therefore in dry weight, occurs soon after fission, giving a curve of cell size more of the type shown in Figure 1*b* (Calkins and Summers, 1941). This may only be an effect of varying degree of hydration, however, and Zeuthen's work (1953) on respiration in *Tetrahymena* suggests that synthesis may be more or less continuous.

Whatever the exact form of the growth curve between one division and the next, it is clear that the processes of mitosis and cleavage are normally triggered off when the size of the cell is roughly double what it was after the previous division. The idea that it is the total size of the cell which somehow provides this stimulus is an old one. It was first put forward by Hertwig (1903) in the form of the nucleo-cytoplasmic ratio, an idea that has been variously acclaimed and criticized (Wilson, 1925). The importance of the ratio of cell surface to cell volume or cell mass has also been stressed from time to time (Berrill, 1943). In this connexion Rashevsky (1938) has suggested on theoretical grounds that diffusion forces might cause a splitting in

* The term 'cell size' is widely used to cover cell volume, total cell mass or weight, and cell dry mass or weight. Such a portmanteau definition may be permissible as a convenience, but it must be emphasized that the most satisfactory measure of individual cell growth is probably dry mass or weight. In certain circumstances it may be desirable also to measure wet weight or degree of hydration.

two when the cell reached a certain size, but the idea has not met with much enthusiasm, perhaps because it seems to neglect the visible machinery of mitosis and cleavage.

If growth is responsible for starting off the processes of mitosis and cleavage, any substances which interfere with the syntheses that underlie growth should appear to be inhibitors of division. It is possible for instance that some of the so-called prophase inhibitors of mitosis are in fact inhibitors of growth. If so, it might be expected that the substances in question would not be effective in stopping mitosis in cells where there was no growth, e.g. totally cleaving eggs. Inhibitors that are effective on the one type of cell, however, seem in general to be equally effective in inhibiting

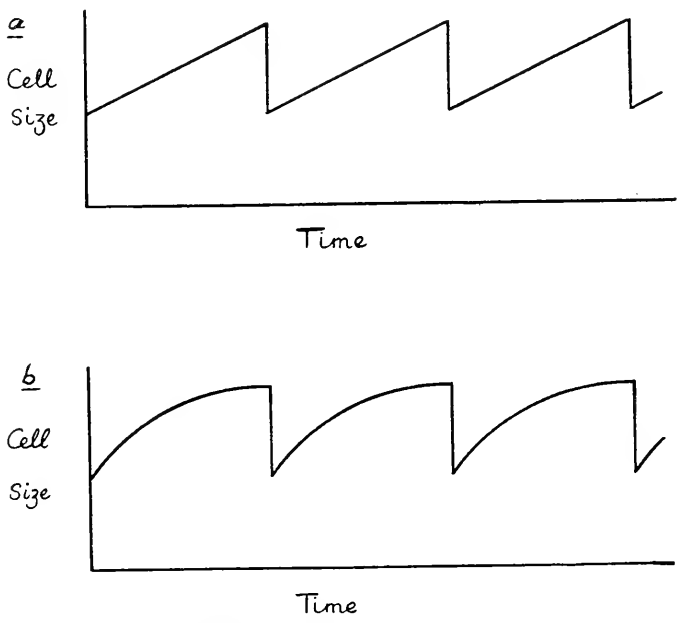

Figure 1. *Possible types of cell growth curve; (a) growth at a uniform rate; (b) growth most rapid immediately after division.*

the other type, though a search of the literature in this field might bring to light some interesting exceptions.

From what has been said above, it will be apparent that there are no very solid grounds for supposing that division is triggered off when cell size reaches a certain level. The fact that, for a given cell type, size may vary over a considerable range, and that most if not all inhibitors of division act alike on cells whether or not they are growing, might suggest, on the contrary, that growth and division are not very closely linked together. At present, all too little is known of individual cell growth in relation to division. But two new techniques, namely the measurement of the reduced weight of cells, using the Cartesian diver, and the estimation of cell dry weight by means of the interference microscope, now offer the possibility of great advances.

DIVISION WITHOUT GROWTH

Until we have more definite evidence along these lines, there is much to be said for considering the control of division in cells where growth is not simultaneously involved, that is to say in the early stages of totally cleaving eggs. Even here of course it is possible that there is some protein synthesis going on, but it can hardly compare with what occurs during the growth and division of ordinary cells.

Mainly because of their convenience as experimental material, sea-urchin eggs especially have been used in the study of division. Much is known about their mechanisms of mitosis and cleavage, and a certain amount about associated chemical changes. This information is mainly derived from a study of inhibitors. In many cases, of course, the chemical action of the inhibitor is uncertain, or even unknown, and the light thrown on the underlying mechanisms of division is therefore not very great. In some cases, however, the action of the inhibitor has been studied in relation to the cell's general metabolism. A certain amount is known, in consequence, about the relation between respiration, the supply of energy and the division process. This work has been reviewed by Krahl (1950).

The processes of mitosis and cleavage involve mechanical work, so it is not surprising to find that division is very dependent on a supply of energy from respiration. In the case of the sea-urchin egg at least it appears that anaerobic glycolysis by itself cannot supply sufficient energy for division. The eggs will not enter division in oxygen tensions below about 0·4 per cent., nor in the presence of inhibitors of the cytochrome system (e.g. carbon monoxide, cyanide, azide) or inhibitors of the Krebs cycle (e.g. malonate). In this respect they differ from various other eggs, and certain somatic cells, no doubt because their powers of glycolysis are only slight*. Again, as might be expected, neither sea-urchin eggs, nor any other type of cell, will enter division in the presence of agents such as dinitrophenol, which interfere with phosphorylation. There is some evidence, however, that once division is under way it is not inhibited by the normal respiratory or glycolytic poisons. The implication would seem to be that the energy for division is stored up beforehand.

In spite of this work on the respiratory requirements of division, very little is known about *when* the energy-producing mechanisms are actually required for the division process. Attempts have of course been made to find variations in respiration during the division cycle, but even the most refined modern techniques of Zeuthen (1950) and Scholander *et al.* (1952), have shown only minor fluctuations in oxygen consumption, and in many cases, no fluctuations at all. The conclusion seems inescapable that either the energy for division is required in a practically continuous flow, or it absorbs so small a part of the total energy output of the cell as to be scarcely detectable.

EXPERIMENTS WITH CARBON MONOXIDE ON DIVISION IN SEA-URCHIN EGGS

With a view to finding out rather more about how the energy-producing mechanisms support cell division, experiments have been carried out on synchronously dividing eggs, using carbon monoxide as an inhibitor of respiration (Swann, 1953).

* About 5 per cent. of the sea-urchin egg's ATP supply is derived from glycolysis (Cleland, 1953).

By taking advantage of the fact that the carbon monoxide-cytochrome oxidase complex is only stable in wavelengths of light outside its absorption bands, it is possible, merely by altering the wavelength of the illuminating beam, to switch on and off the inhibition of respiration while actually observing the eggs. In this way, the eggs were inhibited for varying lengths of time at different points of the division cycle, and photographed by time-lapse, so that their average time of cleavage could be worked out.

The results show that if inhibition is applied before a certain critical point, which occurs at normal temperatures between about 35 and 40 minutes after fertilization, the first cleavage is delayed by a time roughly equal to the duration of the inhibition. If however the inhibition is applied after this critical point, but before cleavage is complete, then the first cleavage is unaffected, but the ensuing cleavage is delayed, by a period again equal to the duration of the inhibition.

The best explanation seems to be in terms of a reservoir mechanism. We may suppose this reservoir to be filling steadily as a result of respiration, and to siphon out when it is full, at about 35–40 minutes after fertilization. This starts off the division process, which continues regardless whether the reservoir is then filling or not. In the normal course of events, however, the reservoir would begin refilling at once, and continue filling during division and the next interphase. At about 35–40 minutes after the first emptying we might expect the reservoir to be full once more, and to siphon out again, so starting off the second division. The time relations of the second division suggest that this is what happens.

Besides accounting for the observed facts, this scheme explains why the first division takes longer than the subsequent ones. The reservoir has first to fill up, and the division has then to run through. Subsequent divisions, however, can occur at 35–40 minute intervals, namely the length of time taken for the reservoir to fill up, and in fact they do. The scheme also offers an explanation of why there are no major fluctuations in respiration during the division cycle. The energy from respiration is utilized continuously by the reservoir mechanism, and it is only the siphonings out that are discontinuous. It is perfectly possible therefore for the proportion of the cell's energy supply required for division to be a substantial fraction of the whole. Finally the hypothesis explains such experiments as those of Jacoby, Trowell and Willmer (1937) where it was found that tissue culture cells on a maintenance medium could be brought into division by an application of embryo juice, but could only be made to divide a second time by further applications of embryo juice, if these were made *during or after* the first mitosis.

EXPERIMENTS WITH ETHER ON DIVISION IN SEA-URCHIN EGGS

With a view to finding out more about the postulated reservoir from a different angle, a further set of experiments was carried out, in which a narcotic, namely ether, was applied as before to synchronously dividing sea-urchin eggs, at different points in the division cycle, and for varying lengths of time (Swann, 1954). These experiments confirm the conclusions drawn from the carbon monoxide work. They indicate the existence of the same sort of reservoir mechanism, and the same critical point in the division cycle at which the reservoir siphons out.

The results are complicated, and the details must be left to the original paper. It is sufficient to say here that ether appears to have two quite distinct effects on the division process. At a concentration of 1 per cent. it prevents the building up of the mitotic figure, but does not affect the filling of the reservoir. That is to say if it is applied, and then removed, before the critical point, it causes no delay in cleavage. If on the other hand it is removed after the critical point, it causes a delay in cleavage, not equal to the total duration of the inhibition, but only to the duration of the inhibition beyond the critical point. The egg in other words remains blocked at the critical point. At higher concentrations not only does ether block the building up of the mitotic figure, but it reduces the rate of filling of the reservoir as well. At a concentration of 2 per cent. for instance, the rate of filling is halved.

The first effect of ether can therefore be likened to preventing the siphoning out of the reservoir, or to preventing the normal action of the reservoir contents once they have siphoned out. The second effect resembles that of carbon monoxide in reducing and ultimately stopping the filling of the reservoir. It is of some interest to note that 1 per cent. ether, by blocking the cell at the critical point, offers a means of bringing batches of eggs fertilized at different times into synchronous division.

THE NATURE OF THE RESERVOIR

Having arrived, by two quite different routes, at the same hypothesis for the control of division in sea-urchin eggs, it may perhaps be permissible to speculate a little about the proposed mechanism. The compelling question, of course, is the physical and chemical nature of the reservoir. Is it to be regarded as a store of energy, or of some other non-energy-carrying compound? If the latter, it must follow from the carbon monoxide experiments that division itself can function on glycolytic energy alone. The critical experiment must be to test the effect of a range of inhibitors of glycolysis, when mitosis is already under way. The experiments of Hughes (1950), however, indicate that fluoride, which is amongst other things a glycolytic inhibitor, applied under these circumstances to tissue cells, may slow down division but does not actually stop it. More work is needed, however, to be certain on the point.

The evidence would seem to point therefore to the reservoir being a store of energy. This energy might of course be built up in a physical form, such as the oriented protoplasmic structure of the sperm aster. The results of the ether experiments, however, make this seem unlikely, for whereas the lower concentrations of ether can be seen under the polarizing microscope to solate the oriented structures of the cell, they are, as was pointed out earlier, without effect on the filling of the reservoir.

It is natural then to turn to the idea of a chemical store of energy, perhaps of some organic phosphorus compound. There are difficulties, however, in thinking in terms of the best-known of these substances, namely ATP and phosphagen. The amount of ATP, it is generally agreed, does not seem to vary during the division cycle. The amount of phosphagen does, it is true, appear to increase shortly after fertilization, but the time relations do not fit in at all with the reservoir hypothesis (Chambers and Mende, 1953). Moreover both these compounds appear to be freely available to the cell as a whole, as they decrease during anaerobiosis (Barth and Jaeger, 1947; Cleland, 1953). But the postulated store of energy in the reservoir does not get used

up during inhibition; if it did, the delays induced by inhibition would always be greater than the duration of the inhibition, in order that the depletion of the reservoir could be made good. Such objections, however, do not rule out the possibility of other more specific organic phosphorus compounds constituting the postulated store of energy.

THE APPLICATION OF THE RESERVOIR HYPOTHESIS TO DIVISION WITH GROWTH

We are now in a position to consider the reservoir hypothesis in relation to the vast majority of cells where division is accompanied by growth. Since the reservoir fills up not only throughout interphase, but during the preceding division as well, it must be presumed, unless the reservoir mechanism is peculiar to egg cells, that a similar mechanism also operates during interphase and the preceding division in ordinary cells. How might such a mechanism fit in with the growth process; and is it possible that the two mechanisms could run side by side and independently as suggested in the first section?

The issue can perhaps be clarified a little by considering the apportioning of the cell's energy supplies between the various cellular activities. It has long been realized that all activities do not have an equal call on these energy supplies; movement, division and irritability, to mention only three, can all be brought to a halt by partial respiratory inhibition, without affecting the cell's maintenance activities, or at least without affecting them irreparably. There is thus no *a priori* reason to suppose that division and growth will have an equal call on the energy supplies.

In the case of the dividing egg cell, two activities alone presumably absorb all the available supply of energy. The first of these is maintenance: making good the wear and tear on the whole protoplasmic structure, and preserving the salt and water balance of the cell; the second activity is division. It is not difficult to see why the normal processes of selection will lead to maintenance having a prior call over division on the energy supply. Division can be postponed without the organism perishing; maintenance cannot. It is not surprising therefore to find that division is suppressed in any tissue by a measure of respiratory inhibition which leaves maintenance more or less unaffected. In the case of the sea-urchin egg, for instance, division is suppressed by a degree of anaerobiosis, which reduces the oxygen consumption to about 30 per cent. of normal (Krahl, 1950). Under these conditions, however, the cell can survive for long periods. It is conceivable, in the light of this figure, that division requires as much as 70 per cent. of the sea-urchin egg's total energy output, i.e. about twice as much as maintenance.

When the cell is both dividing and growing, the situation is more complex. Growth must be added to the two activities mentioned above and, in some cases, movement and perhaps other activities as well. For simplicity, however, maintenance, growth and division can be considered alone. Once again it seems likely that maintenance will have a first call on the energy supply, and as with eggs, there is evidence that cells can withstand for considerable periods a degree of anaerobiosis or respiratory inhibition that prevents division, and in all probability, growth as well. The difficulty arises over the priority for energy as between growth and division. From the point of view of selection, it would seem that a cell which maintained its growth

when the supply of energy was short, but which did not divide, would be in a better position to survive or to withstand further stringency than a cell which continued to divide at the expense of growth. *In extremis*, for instance, accumulated protein can be used as a source of energy, whereas a division that is past and gone cannot.

It must be emphasized here that an argument of this sort is a biological one that sidesteps the biochemical issues. The exact mechanism that results in maintenance, for instance, having a prior claim on energy supplies, is not known; but in the present context it is not necessary to know. It may be that there is a common pool, in which different activities compete for energy; or it may be that different activities are supplied by different metabolic pathways. In the first case it would have to be supposed that the various activities have differing affinities for the available energy. In the second case the various pathways would have to be differentially sensitive to inhibition or anaerobiosis. In either case, however, the end result is the same from the point of view of the cellular economy.

This question of the respective claims of division and growth on the cell's energy supply is capable, up to a point, of being settled experimentally. It is in fact highly desirable that the effect of varying degrees of respiratory inhibition in slowing down both growth and division should be investigated. Using either the diver technique or the interference microscope this should not present undue difficulties. Meanwhile, however, it may be worth examining in a little more detail what the possible results of such studies might be.

If an actively growing cell is subjected by whatever means to a steadily increasing degree of respiratory inhibition, it would be expected, in the light of what has been said above, that division should first slow up, and then stop. Depending on the extent to which the respective claims of division and growth overlap, this slowing up and ultimate stopping of division should be accompanied by some degree of slowing up of growth. Further inhibition will slow up growth yet more, and finally stop it. By this stage it might be expected that maintenance would begin to be affected, at first reversibly. Further inhibition should affect it more drastically, and in due course, irreversibly.

This sequence of events is illustrated in Figure 2*a* which shows the suggested apportioning of the cell's total energy supply between maintenance, division and growth. As the available energy decreases, the upper regions of this diagram should be imagined as being cut off. At 50 per cent. of the normal energy supply for instance, very little energy is left for division, whereas there is still a certain amount available for growth, and maintenance is quite unaffected.

The interesting aspect of this suggested apportioning of the cell's energy supply is that a degree of anaerobiosis or respiratory inhibition, or partial starvation of energy-yielding foodstuffs, will affect division more severely than it will affect growth. *Though the cell will therefore grow more slowly, it will grow larger.*

This is not the only possible scheme of things, though it is perhaps the most likely. An apportioning on the lines of Figure 2*b* for instance, would give a more extreme effect of the kind described above, since division stops entirely before growth is affected at all. In Figure 2*c*, division and growth are affected equally, and cell size should remain constant under all conditions. In Figure 2*d* growth is affected before division, so that the cell should get *smaller* as the energy supply is reduced.

Unfortunately there is almost no direct evidence, and very little indirect evidence, about the effect on cell size of reducing the energy supply. In the case of ordinary somatic cells, either *in vivo* or *in vitro*, measurements of cell size have, it is true, been made from time to time (reviewed by Hoffman, 1953). But the difficulty, not to say impossibility, of estimating with any accuracy the volume of irregularly shaped cells, together with the considerable uncertainty about their degree of hydration, makes the measurements of little value in the present instance. In the case of micro-organisms the situation is rather better. Volume can be estimated much more

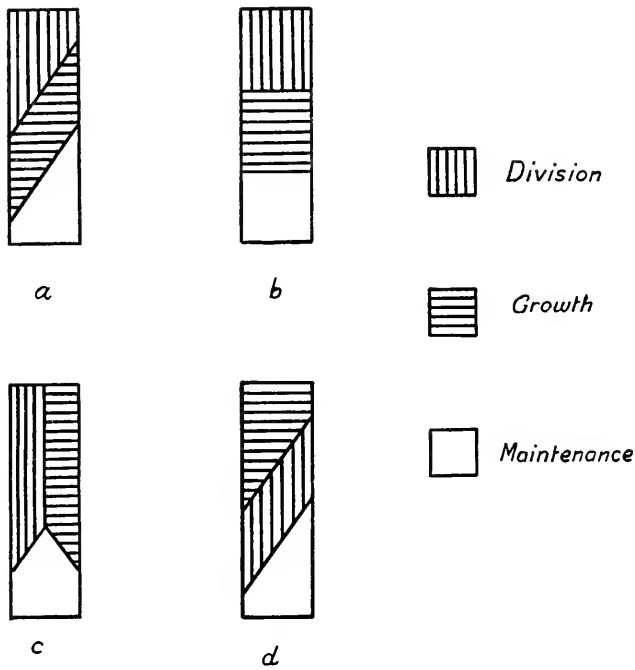

Figure 2. Possible arrangements for the apportioning of the cell's energy supply. For explanation, see text. (a) Inhibition leads to increase in cell size; (b) inhibition leads to increase in cell size; (c) inhibition produces no change in cell size; (d) inhibition leads to decrease in cell size.

accurately, and what is more valuable, the average dry weight of organisms can be determined.

A search of the literature has not brought to light any work on the effect of partial anaerobiosis or respiratory inhibition on cell size in micro-organisms. It has been found, however, by Pace and Ireland (1945), that raising the oxygen tension increases the oxygen consumption but *decreases* the size of *Tetrahymena*. Since increased oxygen consumption presumably means an increased energy supply, the decrease in size is what might be expected from an apportioning of energy of the type earlier deduced and illustrated in Figure 2*a*. It must be remembered however that increased oxygen tension is liable to act as a poison of uncertain effect.

The other references to variation in cell size almost all relate to the growth of populations of one sort of micro-organism or another. The most detailed work on Protozoa is that of Ormsbee (1942) who worked with *Tetrahymena* and found that while oxygen consumption was high during the logarithmic growth phase and decreased in the final stationary phase, cell dry weight behaved in the opposite way. This is what would be expected in the light of the argument above. Such studies have not however always given consistent results (see, for instance, references to earlier work given by Ormsbee, 1942; Calkins and Summers, 1941; Adolph, 1931). But this is not surprising, for although there is general agreement that oxygen consumption and hence, no doubt, energy production falls in the stationary phase (Hall, 1953), presumably as the result of a shortage of energy-producing foodstuffs, numerous other substances that are essential specifically for growth may also run short. More detailed knowledge of the factors underlying the onset of the stationary phase would be needed to get much further along these lines.

The same is true of the other studies on the growth of micro-organisms. There does, however, seem to be good evidence that cell size in yeast is at a minimum during the logarithmic phase and increases during the stationary phase (Richards, 1934). In bacteria too there is wide agreement that cell size varies, though in a different way, being at a maximum in the late lag and early logarithmic phases (Dubos, 1949). But since Robinow (1949) has shown that the so-called cells of this phase are in reality multiple, it seems more likely that cell dry weight is no greater, and perhaps less, at this stage than in the later phases.

The complex conditions of the stationary phase of a culture of micro-organisms are clearly not suited to an investigation of the effect of energy supply on division and growth. If the limiting factor is the energy-supplying substrates, all may be well; but if, as is very likely, other compounds are also lacking, the results will necessarily be confused. Information on the effect of anaerobiosis or respiratory inhibition on both growth and division is therefore required during the logarithmic phase. Such studies should present no special difficulties given the new techniques mentioned earlier.

CONCLUSION AND SUMMARY

There is no direct evidence about the way in which division is controlled in cells that are also growing. The tendency has been to think in terms of division being triggered off in some way when the cell has grown to a certain size, but there are a number of objections to such an idea.

It would appear from the experiments on sea-urchin eggs with carbon monoxide and ether that division in cells without growth is controlled by a continuously operating reservoir mechanism. The preparations for a division are in fact going ahead not only during the previous interphase, but during the previous division as well.

Such a mechanism may of course be peculiar to cells that are not growing. But it is possible at least that a similar mechanism exists in cells that are growing, in which case it would seem that growth and division must be two separate processes functioning simultaneously and more or less independently. If this were so, the relative constancy of cell size would be due simply to the fact that the two mechanisms operated at more or less constant rates. Size however does not remain completely constant

for any given cell type, and this could readily be explained as a slowing up of one or other mechanism.

In an attempt to follow the implications of this idea, the concept of differing 'priorities' for the cell's supply of energy is introduced. It is suggested on purely biological grounds that the first priority is likely to be for maintenance, with growth second and division third. If so, a reduction in the available supply of energy, whether caused by anaerobiosis, inhibitors, or partial starvation of energy-providing foodstuffs, should lead to a slowing down of division before it leads to a slowing down of growth. Such unfavourable conditions should therefore result in an increase of cell size (dry weight). Such evidence as is available suggests that this is the case.

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Discussion

Chairman: H. V. Brøndsted

M. Westergaard. Has it been possible to fix the eggs and see how inhibition affects the differentiation of the chromosomes during mitosis? It seems a pity that the physiological approach to the study of mitosis has a tendency to become detached from microscopic information about the chromosome cycle.

M. M. Swann. The sea-urchin egg is not a very satisfactory material for chromosome studies, and I have only made a few observations. Clearly, more ought to be done along these lines.

E. Zeuthen. I have found that the ^{32}P uptake during mitosis varies cyclically (Zeuthen, 1951, *Publ. Staz. zool. Napoli* **23**, Supplement, 47–69). This might perhaps be evidence of the building up of a store of some phosphorus compound.

M. M. Swann. Agreed.

C. D. Darlington. Indications that DNA is an important component of Professor Swann's 'reservoir' are of several kinds. Cells when x-rayed may be forced into mitosis too soon and produce half-size chromosomes presumably with a half charge of DNA. Their prophases may also be reversed so that when they return to mitosis they have a double set of chromosomes, certainly with double DNA (Darlington and La Cour, 1945, *J. Genet.* **46**, 180–267.) In the differentiation of the bone-marrow, red precursors with a high mitotic rate are marked by a strong DNA charge on the chromosomes, high spiralization and a compact and effective spindle. White precursors with a low mitotic rate have a low DNA charge, low spiralization and a hollow and less effective spindle. This DNA-plus-protein contrast is exaggerated with pernicious anaemia, where it leads to the formation of ineffective red cells lacking a balanced chromosome complement (La Cour, 1944, *Proc. Roy. Soc. Edin.* **62**, 73–85). In pollen grains there is normally a differentiation, determined by a cytoplasmic gradient,

between nuclei which will and will not divide. Again this is correlated with DNA charge and protein supply and can be upset or reversed experimentally (in *Sorghum*, Darlington and Thomas, 1941, *Proc. Roy. Soc.* B **130**, 127–150; in *Tradescantia* and *Scilla*, La Cour, 1944, *Heredity* **3**, 319–337). On the other hand cells can be made to divide with an insufficient DNA charge on the chromosomes which have not reproduced (Beadle, 1933, *Cytologia*, **5**, 118–121, in *Zea mays*). There are therefore alternative and competing stimuli.

F. J. Ebling. I should like to offer some evidence of a different kind in support of Professor Swann's view that cell growth and cell division are independent. During the oestrous cycle of the female rat there are significant changes in thickness of the stratum germinativum but not in the incidence of cell division. It appears that the rate of keratinization alters independently of the incidence of mitosis.

On suction in Suctoria

by

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INTRODUCTION

THE Suctoria are a group of carnivorous Protozoa having tentacles by means of which they hold and apparently suck their prey. I wish to discuss the mechanism of suction.

A suctorian tentacle normally consists of a sheath and an inner tube. The sheath is apparently continuous with the surface of the animal, whereas the inner tube usually extends a little way into the body of the animal, where it ends abruptly (Collin, 1912; Noble, 1932). There is a bulbous or sucker-like expansion at the tip of the tentacle, of a structure not yet convincingly described; in *Tokophrya infusionum* minute papillae have been detected at the tip of the tentacle with the electron-microscope (Rudzinska and Porter, 1953).

Some species of Suctoria are particular as to their food. For instance *Podophrya fixa* is only known to feed on hypotrich ciliates (Collin, 1912, p. 253), and *Discophrya collini* (Root, 1915, as *Podophrya collini*) on holotrichs. Others, however, such as *Tokophrya infusionum*, ingest a wide variety of Protozoa, including both flagellates and ciliates (Iziumov, 1947). In some Suctoria the tentacles contract and extend vigorously; in others the movements are extremely slow and for most of the time the tentacles are motionless. When a suitable food organism touches the tip of one or more tentacles, it is held. Sometimes it breaks away, but if it fails to do so within a minute or so it falls victim to the suctorian. Some species of Suctoria appear to paralyse their prey, as though by some toxic agent. The contents of the prey then pass up the tentacles into the suctorian, the ingested material being held in food vacuoles (Noble, 1932; Rudzinska and Porter, 1953), in which it is presumably digested.

THE HYDROSTATIC GRADIENT

A rough estimate of the force necessary to drive the contents of the prey into the suctorian predator can be derived from the rate of flow up the tentacles by means of Poiseuille's formula. This estimate could be based on the decrease in volume of the prey, but this is difficult to measure accurately. The increase in volume of the suctorian is not by itself a sufficient index of the flow of material up the tentacles, because some of the water so derived is evacuated by extra activity of the contractile vacuole. However, the rate of flow up the tentacles can be estimated from the sum of the increase in volume of the suctorian and the extra vacuolar output. From estimates derived in this way for *Discophrya piriformis*, and on the assumption that

the diameter of the inner tube of the tentacles is 0·5μ, that three tentacles are active, and that the tentacles are 25μ in length, it was calculated (Kitching, 1952*a*) that for a viscosity twice that of water a pressure difference of about 1 cm. of water would be needed. If the viscosity of the food material is greater, the pressure required would be proportionately higher.

THEORIES OF SUCTION

Although the positive hydrostatic pressure within the prey itself might provide the necessary force, it is clear that in the feeding of Suctoria a suction must be exerted. Complete flagellates have been observed inside *Tokophrya cyclopum* (Collin, 1912). The ingestion by *Tokophrya infusionum* of whole ciliates, nucleus, pellicle, and all, has been watched and described by Iziumov (1947). Finally, according to detailed observations by Collin (1912, p. 261), in confirmation of Hartog (1901), the suctorian *Choanophrya infundibulifera* sucks in particles of crushed *Cyclops* from a distance, like a miniature vacuum-cleaner. There is no doubt that Suctoria really do suck.

To explain suction, two plausible theories have been suggested, as well as some others. Peristaltic waves travelling down the inner tube of the tentacles could drive fluid along the tentacles into the body (Collin, 1912), or the body surface might actually increase in area and so create a suction (Kitching, 1952*a*).

Waves have actually been seen by Collin (1912, p. 265) travelling down the inner tube of the tentacles of *Tokophrya cyclopum*, *Discophrya steinii*, and *Choanophrya infundibulifera*. On the other hand waves seen by Dragesco and Guilcher (1950) in *Discophrya piriformis* proceeded in both directions and appeared to them to be inadequate to explain the process of feeding. They also failed to find waves in the tentacles of *Dendrocometes paradoxus*.

The possibility of an active increase in the surface area of the body was suggested by some observations made during the course of experiments on the effects of feeding on the activity of the contractile vacuoles of *Discophrya piriformis*. I noticed that occasionally the body surface of the suctorian became wrinkled soon after the capture of a ciliate, and that later, with the uptake of food into the body, the wrinkles disappeared. This wrinkling might be due either to a decrease in volume of the body or to an expansion of the body surface. Photographs showed that, at a stage when just enough food had been taken up almost to cause the wrinkes to disappear, the body was considerably larger than it had been before the wrinkling occurred. It therefore appeared that the wrinkling was due at least in part to an increase in surface area. It is true of course that there might also have been a temporary decrease in body volume, such as might be produced by an injection of material into the prey, but there is no positive evidence in favour of this view, nor will it by itself explain the observations.

It was found possible to induce wrinkling regularly in *Discophrya piriformis* which had been grown in dilute sea water (5 or 10 per cent.) by feeding it on *Paramecium* in a partially dehydrated state. The *Paramecium* was transferred from a freshwater culture to the same dilute sea water a few minutes before it were offered to the *Discophrya*, so that it had lost water by oxosmosis. The *Discophrya* became wrinkled within a few minutes, and remained so for an hour or more, but ultimately filled up with food so that the wrinkles disappeared. When the wrinkles were about to

disappear the body was considerably larger, as seen in profile, than it had been originally. Although again it might be suggested that the wrinkling was due in part to a loss of material to the shrunken *Paramecium*, there is no positive support for this view, and there is no doubt that the body surface expanded. It seems likely that the attachment of the tentacles to the prey activates the suctorian to an expansion of the body surface which makes room for the uptake of food.

EFFECTS OF HIGH HYDROSTATIC PRESSURE

The interpretation of the expansion of the body surface has been carried a stage further by a study of the effects of high hydrostatic pressure (Kitching, 1954). Experiments have been carried out with pressures ranging up to 15,000 lb. per sq. inch (1,020 atm.). At pressures of 2,000 lb. per sq. inch (136 atm.) and over, the body surface wrinkled. This wrinkling developed in from half a minute to one minute at the lower pressures, but took place within a few seconds at the higher pressures. There was also a tendency for the cuticle to separate from the underlying protoplasm, and for the protoplasmic surface to become rounded within the cuticle. At the higher pressures this happened at the same time as the wrinkling, but at the lower pressures it was often delayed, and did not always occur. At a pressure of 2,000 lb. per sq. inch (136 atm.) the body surface often became smooth again even while the pressure was still maintained, but at 3,000 lb. per sq. inch (214 atm.) this did not happen.

Compression of the contents of the body cannot account for wrinkling. Water is only compressed by about 4 per cent. at a pressure 15,000 lb. per sq. inch, and it is not likely that the presence of proteins or other cell constituents would make any very great difference; nor is there any gas phase in the body. Moreover, the wrinkling took place rather slowly at the lower pressures, and was never reversed immediately on release of the pressure. From photographs taken before and during the application of pressure, it is clear that wrinkling involves an increase in the length of the perimeter of the organism as seen in a sagittal profile, and this implies an increase in the surface area of the body. This conclusion has received support from the results of recent experiments in which a relatively low pressure (2,750 lb. per sq. inch) was applied to *Discophrya piriformis* in the process of feeding. This pressure was sufficient to cause wrinkling but not to prevent feeding. By the time enough food had been taken in almost to fill up the wrinkles, the body was considerably larger than it had been before the application of pressure.

In those experiments in which the pressure applied was relatively low, the protoplasm often remained in contact with the expanded cuticle (Kitching, 1954, Plate I), so that it is necessary to conclude that the protoplasmic surface expanded also. The rounding up of the protoplasm which occurred at the higher pressures may be compared with the rounding up of an *Amoeba* or of a dividing *Arbacia* egg at similar pressures (Marsland and Brown, 1936; Marsland, 1938).

On release of pressure, the protoplasm, if separated from the cuticle, often spread back into contact with the latter within the following few minutes or less. This movement must involve either an increase in volume of the protoplasm or an increase in the wrinkling of the pellicle. It cannot be ascribed to an increase in

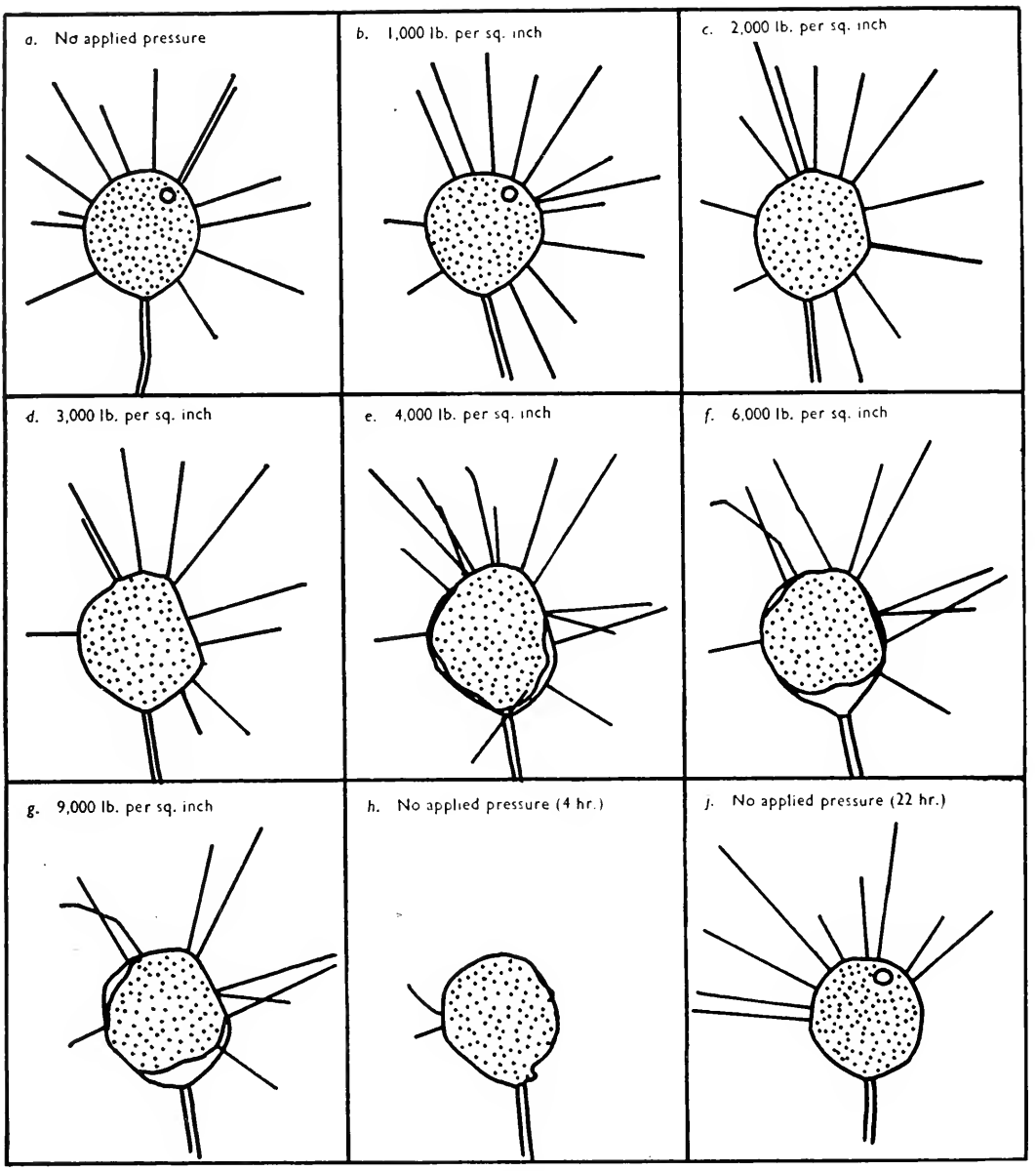

Figure 1. *Effects of hydrostatic pressure on the suctorian* Discophyra piriformis*. *The specimen shown was subjected to increasing pressures step by step, with from* 10 *to* 20 *min. at each step. The drawings illustrate the condition attained near the end of each period of exposure, when any further change was very slow. The drawings have been traced from photographs.*

*This species is very close to *D. collini* (Root) and possibly identical with it.

volume of the protoplasm due to decompression, as it takes place too slowly. Although in some cases there was an indication of a swelling, some time after release of pressure, associated with a prolonged stoppage of the contractile vacuole, it is by no means clear that this accounted for the rather rapid spread of the protoplasmic surface which was sometimes seen.

The cuticle remained wrinkled for many hours after the release of pressure. However the wrinkles slowly disappeared and eventually cuticle and protoplasm became rounded in outline. Wrinkles formed at 2,000 lb. per sq. inch were observed to disappear even while the pressure was maintained, and in other cases in which limited wrinkling was induced by rather brief exposure to high pressure the wrinkles disappeared within a few minutes after release of the pressure. It may therefore be suggested that there is a mechanism by which the fit of the pellicle to the protoplasm is constantly subject to adjustment. This mechanism resides in the protoplasmic surface and is likely to be enzymatic. It was noticeable that after release of pressure, if there was some portion of the pellicle which was not brought into contact with the protoplasmic surface by the spread of the latter, this portion did not become remodelled, but persisted as an excrescence. Mechanisms for the adjustment of pellicular fit must be widespread in the Protozoa. The tendency of the protoplasmic surface to expand when the cuticle allows it to do so is evidently opposed by high pressure, and only reaches its full development after the pressure has been released.

FEEDING IN SUCTORIA

Let us now return to the process of feeding in Suctoria. There is clearly a process of activation. In *Choanophrya infudibulifera* suction is not provoked by carmine particles, but is initiated by genuine food (Collin, 1912). In *Discophrya piriformis* the expansion of the body surface follows attachment of the tentacles to the prey. The expansion of the cuticle is somewhat reminiscent of the formation of a fertilization membrane, although it is carried out without the elevation characteristic of the latter. The cuticle of *Discophrya* is probably also made of protein. It is not clear whether expansion of the cuticle merely permits expansion of the protoplasmic surface within it, or whether activation directly stimulates the protoplasmic surface to expand. It is perhaps relevant that, on the application of pressures of 10,000 lb. per sq. inch (680 atm.) or more, there was usually an apparently simultaneous expansion of the cuticle and separation from it of the protoplasmic surface, and that on release of pressure the protoplasmic surface spread, and made contact with the expanded cuticle. This shows that expansion of the cuticle can at least occur independently, although it may well be brought about by reactions at the protoplasmic surface. So far I have not been able to induce wrinkling in *Acineta* by the application of high pressure. This suctorian has a very loosely fitting cuticle or case, with plenty of room inside for expansion during feeding, so that presumably a mechanism for the rapid expansion of the cuticle is unnecessary.

It is not known whether expansion of the body surface of *Discophrya piriformis* merely makes room for food material driven in by other means, such as peristalsis of the inner tubes of the tentacles, or whether this expansion actually creates a suction. A more detailed study of the peristaltic movements of the tentacles is

needed, in material favourable for this purpose. It is possible that there may be considerable specific variations in the part played by this form of activity, and in any case the observation needs repeating. Peristalsis of the tentacles would account for the fact that a feeding suctorian becomes nearly spherical when full, as though pumped up. It would no doubt also help in the passage of lumps of material. On the other hand the expansion of the body surface, if accompanied by a resistance to inward collapse, would very conveniently provide a suction. It might take place in the way suggested by Mitchison (1952) for the surface of dividing sea-urchin eggs.

The contractile vacuole plays an important though limited part in the process of feeding (Pestel, 1931; Kitching, 1951 and 1952*b*; Rudzinska and Chambers, 1951; Hull, 1953). It operates at a much faster rate during feeding, and so serves to remove some of the water brought in as part of the substance of the prey. During a meal the suctorian swells much less than the prey shrinks, and the difference is accounted for by the extra activity of the contractile vacuole, (Kitching, 1952*b*; Hull, 1953). If the body volume were constant, and the surface of the body were rigid, extra activity of the contractile vacuole would presumably produce a suction which might account for the process of feeding. However, the body does in fact get bigger, and this cannot be explained by vacuolar activity.

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Discussion

Chairman: H. V. Brøndsted

M. M. Swann. I am interested in the idea of suction generated by expansion in view of my own and Mitchison's work on membrane expansion as the possible mechanism of cell division. In the light of our experiments on the modulus of cell membranes, I think that 1 cm. of water is a possible figure.

I should also like to suggest that a study of the flow up the tentacle with different viscosities of protoplasm might help to settle whether suction is caused by expansion or by peristalsis. A very low viscosity would help the first mechanism, but possibly not the second.

J. A. Kitching. The spherical shape of the organisms at the end of a good meal supports the idea that they have been filled up, as though by peristalsis of the inner tube of the tentacles. However, both mechanisms may operate. On the rather rare occasions when wrinkling occurs during normal feeding, or when it is induced experimentally by the use of a shrunk ciliate for food, the protoplasmic surface certainly expands together with the pellicle; and the same is true in certain of the pressure experiments in which the pressure used was not very high. In these cases the expansion cannot be attributed to an internal pressure caused by peristalsis of the tentacles.

J. F. Danielli. Does the application of pressure stop the inflow of material from the prey?

J. A. Kitching. Pressures above about 4,000 lb. per sq. inch stop the uptake of food. The tentacles attached to the prey, shortened during the feeding process, gradually extend, and the prey is imperceptibly released.

R. J. Goldacre. Can a comparison be drawn between the expansion of the surface of *Discophrya* which occurs during feeding and the expansion of the surface of *Amoeba* caused by fat-solvent anaesthetics?

J. A. Kitching. It is likely that the expansion of the surface of *Discophrya* during feeding is secondary to the expansion of the pellicle. It is however interesting that on release of *Amoeba* from high hydrostatic pressure there is an aggregation of the granular cytoplasm to the centre, and an extrusion of clear liquid beneath the plasmalemma, to give an appearance very like that described by you (Goldacre, 1952, *Symp. Soc. exp. Biol.* **6**, 128–165), for amoebae treated with anaesthetics.

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