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THE CHEMICAL KINETICS OF THE BACTERIAL CELL

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CHEMICAL KINETICS
OF THE
BACTERIAL CELL

BY

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OXFORD
AT THE CLARENDON PRESS

Dr. R. M. Lodge who shared many arduous hours while we taught one another much that we at least found it exciting to know; to a select group of research students who have collaborated in experimenting in and thinking about this subject; to the authorities of Imperial Chemical Industries (Dyestuffs Division); to Dr. W. T. Astbury for permission to reproduce the diagram on page 20: and, as always, to the Secretary and other members of the staff of the Clarendon Press.

I.

OXFORD
1946

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I

INTRODUCTION

1. The problem

NATURE possesses the art of weaving simple threads into patterns of wonderful complexity and beauty. The whole spectacle of the chemical and physical transformations which go on around us evolves out of the operation of a few fundamental laws in a manner of which we now possess at least a general idea. Changes in inanimate matter are usually more or less isolated and independent, but those in living cells depend upon processes co-ordinated in a special manner. To the mind of a physical chemist who has studied the former there must occur the question whether the chemical kinetics of the latter can be understood.

Bacteria constitute the best material for inquiry, being without any grossly differentiated structure, and yet showing most of the major phenomena of life. They consist of single cells, which, in favourable circumstances and when provided with the requisite raw materials—often of the very simplest kind such as ammonia, glycerol, and certain salts—synthesize more of their own substance, increase in size and then divide to form fresh cells. Under suitable conditions this goes on indefinitely: under less suitable ones growth stops and the cells may die. Bacteria contain enzymes capable of bringing about a great variety of chemical changes. When exposed to new conditions of life they show remarkable phenomena of adaptation, being capable for example of 'training' themselves to resist high concentrations of powerful drugs which initially would have inhibited all growth.

It is fascinating to inquire whether the principles of chemical kinetics would predict behaviour of this kind.

2. Applicability of chemistry and physics to cell phenomena

At first sight two tremendous obstacles seem to bar the way to the deeper understanding of living cells. On the one hand, the processes occurring in them appear manifold and complex beyond anything that is encountered in the chemistry or physics of inanimate substances. On the other hand, living matter can be the seat of consciousness, the relation of which to the concomitant material

changes constitutes an unfathomable mystery. On closer approach these obstacles lose some of their formidable appearance.

Multiplicity and complication arise, as often as not, from repetitions and permutations of a few simple elements, and the principles of combination are often discernible even though the results are tangled, as the kaleidoscope and the theory of spectra bear witness. Nor need all the details invariably be known in order to arrive at important general conclusions about phenomena. The dynamical problem of three bodies is insoluble, but the kinetic theory of any number of molecules succeeds: the motives of individuals are inscrutable, even when their actions are known, yet an intelligible history of nations can be written.

As to consciousness, it does not seem to be much in evidence in simple organisms such as bacteria: and even with man there appears to be general agreement that a large proportion of his bodily activities for a large part of the time are automatic. Thus even with the physiology of the higher animals one can probably go a long way without meeting the problem of conscious control, and with unicellular organisms one can almost certainly go nearly the whole way. This does not imply an ultimately materialist view. It means rather that the objective set is limited—though by no means unambitious—in somewhat the same way as one might study musical theory, the structure of music and the rules of composition, without finding it expedient to enter into the nature of the aesthetic or mystical qualities of great music. To study the vehicle, whether of conscious life or of musical aesthetics, independently of what the vehicle may in proper circumstances convey, is not, as is sometimes suggested of men of science, to ignore higher values but merely to show a due sense of what is accessible to a given kind of inquiry.

However this may be, one method of approach to which no valid objection whatever can be raised is to find out whether by the operation of known physical and chemical principles any phenomena analogous to those shown by the living cell can be accounted for. If these prove numerous and striking enough, working hypotheses about cells can be established. And the stages by which working hypotheses develop into theories and accepted truths are too well known to need discussion here. In this way one may hope to understand at least some of the properties of living matter. Only by discovering definitive failures of this method can one hope to see

where and how non-physical principles may exert their control.† Even the part played by the famous Uncertainty Principle (the status of which might be regarded as intermediate between a physical and a non-physical one), and the operation of which in biological phenomena has from time to time been postulated,‡ can only be assessed satisfactorily after a great many working hypotheses about such phenomena have been explored in detail.

The present essay has limited scope which may be set forth as follows. A good deal is now known about the kinetics of chemical reactions,§ homogeneous and heterogeneous, simple and complex. In a cell, sequences of such reactions occur in a precisely co-ordinated manner. The seat of the chemical changes is principally a complex texture of macromolecular substances possessing a spatial organization which must help to determine the time sequence of the reactions. The question is whether, having learnt something about chemical reactions in isolation, one can take a further step and begin to understand the principles according to which they are linked together, spatially and temporally, for the purposes of living cells. To return to the metaphor of music, the problem is whether, having studied isolated themes, one can inquire into the laws by which symphonies are—or even could be—constructed. This particular aspect of the matter constitutes a chapter of chemical kinetics which follows naturally on those which have preceded it.

Before discussing cell phenomena at all, it will therefore be expedient to consider certain relevant characteristics of chemical reactions in unorganized systems.

3. Chemical kinetics

No attempt will be made here to expound the principles of this subject, for which reference may be made to the books cited below.§ But the following may be said. Atoms and free radicals are very ready to react, and require to be supplied with little or no energy to enable them to do so (except that certain radicals have to be supplied with the energy needed for a reorganization of their

† e.g. the general scepticism expressed many years ago in Haldane's *Mechanism, Life and Personality* was a valuable corrective to hasty materialism, but would be unfortunate if applied to discourage detailed inquiry into physico-chemical mechanisms.

‡ See for example E. Schroedinger, *What is Life?* 1944.

§ L. S. Kassel, *Kinetics of Homogeneous Gas Reactions*, 1932; C. N. Hinshelwood, *Kinetics of Chemical Change*, 1940 (1945); H. J. Schumacher, *Chemische Gasreaktionen*, 1938.

bonds, e.g. in CH_3— from a planar form to a tetrahedral form on entry into combination). Molecules in general have to be supplied with activation energy (by suitable collisions or by absorption of light quanta) before they are able to react. Free radicals attack saturated molecules with an intermediate, but usually rather small, activation energy. The magnitude of the activation energy, E, is determined from the influence of temperature on the reaction velocity constant, k, by application of the Arrhenius equation: $d\ln k/dT = E/RT^2$. E is the principal factor determining the range of temperature in which a reaction occurs with appreciable velocity. For reactions to occur in the range characteristic of biological phenomena, E must be comparatively small. At such temperatures, resolution of most molecules into free radicals could not play an appreciable role, unless there were an initial supply of radicals to start the reaction, when it might be maintained by a chain process.

Chain processes are quite common, especially, but by no means exclusively, in gas reactions at higher temperatures. They occur typically in such reactions as the oxidation of hydrocarbons. With hexane, for example, the chain process is appreciable at a temperature as low as 200° C. Certain types of molecular structure specially favour the production of free radicals,† and in appropriate circumstances these probably intervene even in liquid phase reactions at low temperatures.

One of the problems of chemical kinetics is to relate the rate of reaction to the concentrations of the reacting substances. Usually this is quite a complicated problem. For reactions which take place homogeneously in one single stage, an elementary application of the law of mass action suffices, and changes of the first, second, and third order can be distinguished. These, however, are the exception, most chemical reactions being resolved into a series of elementary processes to each of which the law of mass action must be separately applied. Further reference will be made to the methods of calculation employed.

One of the most important classifications of reactions is into the two groups of homogeneous and heterogeneous processes. In the latter the rate is determined, not by the concentration of a reacting substance in a single phase, but by the density of molecules at an interface, such as the surface of a solid catalyst. The equation which

† Cf. A. Kossiakoff and F. O. Rice, *J.A.C.S.*, 1943, **65**, 590.

relates, for a given temperature, the amount of a substance on a surface to its concentration in a gas phase or solution is therefore of great importance. It is called the adsorption isotherm. The form of such equations will prove to be of significance in later sections of this book, and will therefore receive some attention in the course of the present introduction.

Although a detailed discussion of most aspects of kinetics is unnecessary for the present purpose, there are certain matters which will find very direct application in the discussions of cell reactions. It is proposed, therefore, to deal with these specifically in the way which will be most convenient for future reference.

4. Adsorption isotherms. Langmuir isotherm

Let a substance, S, at concentration c in a gas or solution be adsorbed at the surface of another phase (such as a solid catalyst). Consider unit area of the surface and suppose that a fraction σ is occupied by adsorbed molecules of S, $(1-\sigma)$ being left free. There is a dynamic equilibrium between free and adsorbed molecules, and this is expressed by the equation

$$k'c(1-\sigma) = k''\sigma, \tag{1}$$

where k' and k'' are constants. The left-hand side expresses the fact that the rate of deposition of S-molecules on to the surface is proportional to the amount of free surface and to the concentration of the molecules in the gas or solution: the right-hand side, the fact that the inverse process is proportional to the density of molecules on the surface. Rearrangement of (1) gives

$$\sigma = \frac{bc}{1+bc}; \tag{2}$$

where $b = k'/k''$.

If the adsorbed molecules undergo a chemical transformation at a rate proportional to their density on the surface, then

$$\text{rate} = \frac{kc}{1+bc}, \tag{3}$$

where k is a constant.

Equation (2) is known as the Langmuir isotherm. It has the following properties. For small values of c, the amount adsorbed is linearly proportional to c, while for large values the amount adsorbed becomes independent of c. This relation is shown in Fig. 1. From

(2) also follows the result that for large values of c the value of $(1-\sigma)$, that is, the free surface, is inversely proportional to c.

The Langmuir isotherm describes the simplest possible case. It is in fact often a very good approximation for the description of the behaviour of real adsorption systems. But it is based upon several assumptions, and these are not always justifiable. The kinds of deviation which occur are of importance.

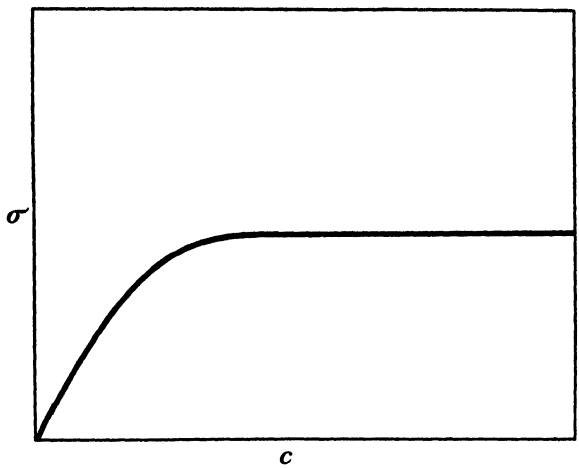


FIG. 1. Langmuir adsorption isotherm.

5. Deviations from the Langmuir isotherm†

In (1) the assumption is made that deposition on the surface is determined only by the free area and by the concentration in the gas or solution, and, correspondingly, that the escape of molecules from the surface into the continuous phase depends only on the number. These postulates are equivalent to the condition that the adsorption or desorption of any given molecule is uninfluenced by the presence of other molecules on neighbouring sites. But in real cases of adsorption molecules exert mutual influences and these are of two general kinds. First, there may appear what is known as a *co-operative effect*, the presence of adsorbed molecules on given sites facilitating the adsorption of molecules on neighbouring sites. This effect depends upon mutual attractions, and plays an all-important part in the phenomena of liquefaction and solidification. It will be

† See S. Brunauer, *Physical Adsorption*, Princeton and Oxford, 1943.

particularly in evidence in adsorption when the surface film can assume a configuration resembling a liquid or solid layer of the adsorbed substance. The form of the adsorption isotherm will be rather complicated, since the energy of adsorption of a new molecule will be different according to whether it goes into a site with one, two, three, or more neighbours. And the variation of the energy with the number of neighbours will itself be a function of the individual molecular structure. The co-operative effect can lead to isotherms

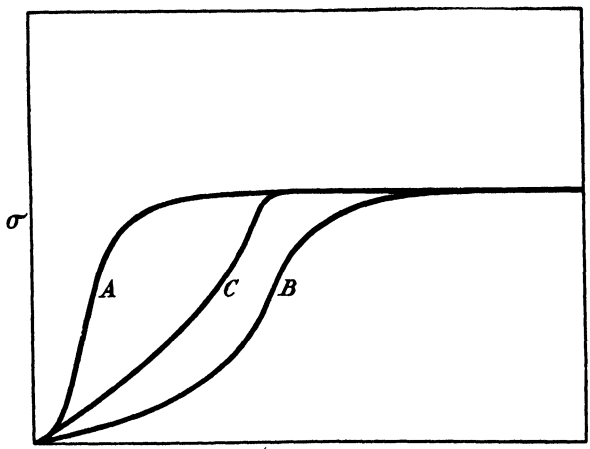


FIG. 2. Isotherms showing co-operative effect.

of the various forms shown in Fig. 2. The expression of these by theoretical equations is no easy matter, and empirical or semi-empirical equations are often employed with advantage. Curve *C* in Fig. 2, for example, might arise if the co-operative effect operated in such a way that nearly complete surface films showed a great tendency to perfect themselves, that is, that the adsorption energy increased very steeply with the number of neighbours. For empirical purposes the curve could probably be well enough represented over the range $\sigma = 0$ to $\sigma = 1$ by an equation of the form $\sigma = ac + bc^n$, c being the concentration, a, b, and n being constants and the exponent n being considerably greater than unity. (This particular equation would have to be used with the convention that it ceased to apply beyond the point where $\sigma = 1$.)

Secondly, there is an effect which acts in opposition to the above, and which consists in an interference by molecules already present

with the adsorption of other molecules on neighbouring sites. This effect may in part be referred to repulsive forces between molecules, and, in so far as this is possible, bears the same relation to the co-operative effect, as the b term of van der Waals's equation bears to the a term. But another factor also enters (and is of special interest in connexion with the phenomenon of the poisoning of catalysts). The basis of adsorption is an attractive force between atoms in the surface of the adsorbent and atoms in the molecules which are adsorbed. Thus we have a whole array of adsorptive sites spaced, in the case of a solid adsorbent, in a regular geometrical pattern, and these may engage either one or several centres of attraction in the molecules of the adsorbable substance. Now if molecules are held by multipoint adsorption and occupy several sites, the possibilities of mutual interference are more serious than if they occupy single ones only. If adsorption occurs on single sites, then, when only one of these remains free, that one is still available for use. But if adsorption occurs in such a way that, say, six sites are used for each molecule, then very large numbers of free sites may remain, but be quite unavailable, being grouped in unfavourable configurations which do not leave six properly related ones clear.† In such cases as this there will be departures from the Langmuir isotherm opposite in sense to those caused by the co-operative effect.

With the two major effects above described acting in competition, very varied shapes of adsorption isotherm can arise, as illustrated in Fig. 3. By a compensation of opposing effects it is not uncommon to have a curve which is nearly linear almost up to the saturation point.

In studying the inhibitory or anticatalytic actions of adsorbed substances on a surface reaction we are not so much interested in σ as in $(1-\sigma)$. The general course of this function is of course quite obvious from that of σ, but it will be useful for future reference to call attention to one or two specific cases. When the isotherm is linear nearly up to the saturation point, then the rate of the inhibited reaction, being proportional to the free surface, will decrease linearly almost to zero with increase in the concentration of the inhibitor. We shall encounter this case in connexion with certain drug actions. A second kind of case is illustrated by Fig. 4. The dots represent adsorption sites on a surface. A is a molecule occupying six, B a

† Cf. E. F. G. Herington and E. K. Rideal, *Trans. Faraday Soc.*, 1944, **40**, 505.

molecule occupying one only. Suppose A is the inhibitor of a reaction which occurs to some other molecule in the adsorbed condition. The

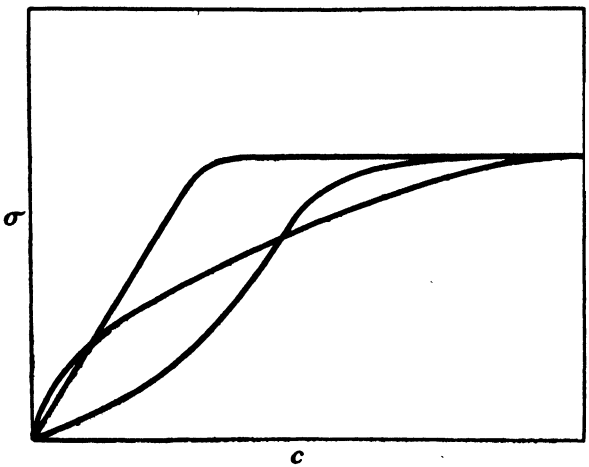


FIG. 3. Isotherms showing opposing effects.

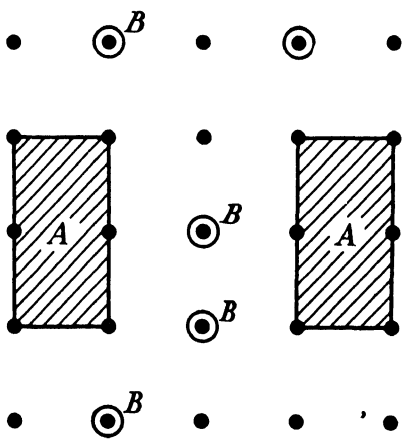


FIG. 4. Blocking of adsorption sites by molecules.

two molecules of A shown in the diagram leave no space for the adsorption of further A molecules. The inhibitory action of A must therefore have reached its limit. If the reacting molecule were comparable with A in size and configuration, its adsorption would also

be prevented and the reaction rate would have been reduced to zero. If, however, the reacting molecule occupied a much smaller space, as *B* in the diagram, then there would still be plenty of room for it in the interstices. Thus the very quality of *A* which gives it a powerful inhibiting action in small concentrations presently brings its action to a limit while there is still a finite reaction velocity.

6. Adsorption isotherms and cell phenomena

Reactions in cells must partake to a considerable extent of the nature of heterogeneous reactions. Some of them will occur with adsorbed substances on the surface of enzymes: and even where it is hardly legitimate to think of a definite surface, the seat of reaction will consist of sheets or networks of macromolecular substances. Such a system, even when permeable in three dimensions, will present arrays of active centres which can be regarded as equivalent to those considered in the derivation of the Langmuir isotherm or its elaborations. Soluble and diffusible substrates before they can react with, or under the influence of, the cell substance must enter into a relation with it which is mathematically the same as adsorption.

One of the outstanding characteristics of adsorption systems is their saturability, and accordingly we should expect to find reaction rates proportional to substrate concentrations for very low values of the latter and independent of them for higher values. Such relations are in fact very much in evidence in cell phenomena.†

The manifold inhibitory actions of antibacterial substances and drugs we should expect to be governed, in part, by the considerations of the previous section. The extent to which this is so will appear in due course.

7. The separation of complex reactions into stages

It has become clear that chemical reactions are always resolved into a series of relatively simple stages. The reaction $2H_2+O_2 = 2H_2O$, for example, does not take place in the manner suggested by the equation, but in a series of steps, such as $H_2 = 2H$, $H+O_2 = OH+O$, $OH+H_2 = H_2O+H$. In these nothing more elaborate occurs than the breaking of one bond or the removal of one atom at a time.‡

† Compare p. 68.

‡ C. N. Hinshelwood and A. T. Williamson, *The Reaction between Hydrogen and Oxygen*, Oxford, 1934; B. Lewis and G. von Elbe, *Combustion, Flames and Explosions of Gases*, Cambridge, 1938; A. H. Willbourn and C. N. Hinshelwood, *Proc. Roy. Soc.*, 1946. A. **185**. 353.

The action of nitric acid upon metals, for which it used to be conventional to write equations of the type

$$3Cu+8HNO_3 = 3Cu(NO_3)_2+2NO+4H_2O$$

and

$$Cu+4HNO_3 = Cu(NO_3)_2+2NO_2+2H_2O$$

can in fact be interpreted as a series of elementary processes of which those are the most probable which involve the fewest molecules and the minimum of simultaneous rearrangements of atoms.†

In polymerization and polycondensation reactions macromolecules of enormous size are built up, but always in a series of steps. The polymerization of ethylene, for example, is initiated by a free radical which reacts as follows:

$$R— + C_2H_4 = RCH_2CH_2—.$$

The new radical reacts with ethylene again,

$$RCH_2CH_2— + C_2H_4 = RCH_2CH_2CH_2CH_2—,$$

and there ensues a series of additions which, according to circumstances, may stop at two or three or proceed to hundreds or thousands.

For the purpose of subsequent discussions it is convenient here to emphasize certain consequences of this resolution of chemical reactions into stages.

(*a*) The fact that the individual steps are usually very simple, but may be combined in different ways and independently influenced by conditions such as temperature and concentration, leads to the result that the *same basic mechanism may yield a considerable diversity of products and in very varied proportions.* This gives the appearance of a greater complexity than is really inherent in the nature of the system.

(*b*) The existence of transitory intermediates *renders possible the energetic coupling of reactions which, according to the stoichiometric equations, appear to be independent.* We may have the two reactions

1. $B+A_2 = BA_2$ with diminution of free energy.
2. $XY = X+Y$ with increase of free energy.

If the diminution of free energy in the former exceeds the increase in the latter, there is no thermodynamic reason why the two reactions

† U. R. Evans, *Trans. Faraday Soc.*, 1944, **40**, 120.

should not be so coupled as to allow one to provide the free energy for the other. But the mechanism by which the requisite coupling can occur is at first sight obscure. It becomes quite clear, however, when the resolution into steps is considered. The series of changes:

$$\begin{aligned} A_2 &= A+A \\ A+XY &= AX+Y \\ AX+B &= AB+X \\ AB+A &= BA_2 \end{aligned}$$

is equivalent in final result to the two individual reactions 1 and 2.

(*c*) *The individual elementary steps of a complex mechanism are sometimes spatially separated.* One of the commonest examples of this is provided by a type of gaseous chain reaction, where certain radicals are generated initially at the wall of the vessel, undergo a series of intermediate transformations in the gas phase, and meet their final fate by diffusion once more to the wall. In some such reactions there will be established at a given moment not only definite concentrations of various intermediates at each given point of space, but definite concentration *gradients.*

Some of the intermediates in oxidations, decompositions, and so on are free radicals or molecules of very short life, and the overall course of the change depends upon the space-time relations in a variety of ways. Only one simple example need be quoted here. When a stream of radicals is produced by the pyrolysis of, say, ether at a high temperature and is pumped at low pressure along a tube with a thin metallic mirror of tellurium deposited on its wall, then, if the distance from the source of the radicals to the mirror is small, methyl radicals combine with the tellurium and remove it. If, however, the distance is increased, there is no removal, because the radicals suffer alternative fates before having time to reach the metal.

The minute size of bacterial cells makes it possible in principle that highly unstable intermediates formed in one region of the cell would be able to diffuse to another region and there enter into further reactions without necessarily suffering destruction on the way.

8. Stationary states

When certain intermediates in a complex reaction series are labile and short-lived, then there is rapidly established a stationary state (quite distinct from an equilibrium), the nature of which is best

illustrated by an example. Suppose a chain reaction occurs according to the following scheme (which is a rather rough over-simplification of what happens in the union of hydrogen and chlorine in presence of oxygen):

$$1. \quad Cl_2 = 2Cl$$
$$2. \quad Cl + H_2 = HCl + H$$
$$3. \quad H + Cl_2 = HCl + Cl$$
$$4. \quad Cl + O_2 = ClO_2.$$

Chlorine atoms and hydrogen atoms are transitory intermediates, the concentrations of which adjust themselves very rapidly to satisfy the equations

$$d[Cl]/dt = 0 \quad \text{and} \quad d[H]/dt = 0; \qquad (4) \text{ and } (5)$$

that is to say,

$$F_1 - k_2[Cl][H_2] + k_3[H][Cl_2] - k_4[Cl][O_2] = 0 \qquad (6)$$

and

$$k_2[Cl][H_2] - k_3[H][Cl_2] = 0, \qquad (7)$$

where F_1 is the rate of process 1 and k_2, k_3, and k_4 are constants.

The actual rate of formation of hydrogen chloride is obtained by writing down the expression for $d[HCl]/dt$, which, of course, is not equated to zero. Elimination of [H] and [Cl] gives the required result.

The concentrations of chlorine and hydrogen change progressively, and difficulty is sometimes felt about the equating to zero of the net rates of formation of the atomic species. The justification is that the steady state to which this equation corresponds is established so rapidly that no appreciable change in the concentrations of the molecular species occurs in a comparable time, and that the solution of (6) and (7) gives the concentrations of atoms corresponding to these particular constant values of $[H_2]$ and $[Cl_2]$. In so far as the latter slowly change, then the atomic concentrations change with them, but always in accordance with (6) and (7). In relation to the rapid processes envisaged in the establishment of the conditions (4) and (5) changes in the molecular concentrations have the character of secular variations of constants.

These principles will almost certainly find application in the kinetics of cell processes. Such processes will necessarily involve labile intermediate substances produced and consumed at rates which, referred to unit concentration, are enormous compared with the changes in concentration of the primary sources of growth material.

9. The scale effect

The *scale effect* depends upon the simple fact that if we change the linear dimensions of an object in a given ratio, then the ratios in which we change its area and its volume respectively are different from that and from one another. This means that a smaller scale model or an enlarged version of any system can seldom give a true representation of it in more than a limited degree. If a painter represents a building by scaling down its linear dimensions 500 times, this may satisfy the eye of an observer who is concerned with linear ratios such as relative heights and widths of windows. But areas are scaled down in a different ratio, namely $(500)^2$ and in some ways, for example, in connexion with effects of light and colour, this changes the impression made by the original. To correct this the painter may resort to what by the simple linear standard of comparison would be called distortion.

In physical chemistry the scale effect is profoundly important, and chiefly for the following reasons. The amount of a homogeneous chemical change occurring in a system, or the amount of heat generated or absorbed is proportional to the volume: the rate at which matter or heat can enter or leave the system is proportional to the area of its bounding surface. There is, for a given geometrical form, only a single value of the volume for which the ratio of volume to area has an assigned magnitude. Suppose, for example, that an exothermic chemical reaction occurs in a spherical vessel of radius r, and that the heat generated is removed by conduction through the surface. A temperature distribution is established such that the rate of generation of heat by reaction equals the loss by conduction. Now suppose the same reaction to occur in a vessel of radius $10r$. The amount of heat generated is now 1,000 times as great, but the area through which conduction can occur is only 100 times as great. The original thermal state cannot be reproduced, but the temperature must rise. This kind of consideration gives rise to great difficulties in translating laboratory experiments to the technical scale. In some cases actual discontinuities may arise as the size of the system increases. A good example is found in the various branching-chain reactions which exhibit explosion limits. Here branching of reaction chains (dependent upon the volume) is opposed by deactivation processes (occurring at the wall and dependent upon the area). At a certain critical size the latter factor can no longer balance the

former and explosion occurs.† The various examples of this discovered among oxidation reactions of phosphorus, hydrogen, and other combustible substances were the chemical prototypes of the even more spectacular branching-chain disintegrations of uranium and plutonium.

In a cell there occur synthetic and other chemical processes which must be more or less proportional in amount to its volume. The raw materials for these reactions must arrive from the external medium through the cell wall and waste products must diffuse out by the same route, and all at rates which are more or less proportional to the surface area. As the cell grows the scale effect comes into operation. Substances formed by the cell must increase internally in concentration, while those derived from outside must tend to drop.‡ More subtle local changes in concentration and in concentration gradients will also occur as the scale of the internal fine structure increases. At a given size of cell, certain concentrations will rise above or fall below what may be a critical value. In a quite general way we see that factors of this kind may well be responsible for issuing the command at which the onset of cell division occurs.

10. The building up of ordered crystalline structures

Living matter reproduces itself, and, complex though its structure may be, it possesses some of the characteristics of crystallinity. Various proteins, enzymes, and viruses can in fact be obtained in definitely crystalline forms.§ Enzymes are not themselves living, but constitute essential parts of living cells. It is therefore relevant, while remaining on one's guard against facile analogies, to keep in mind some of the main principles governing the growth of crystals, which are themselves capable of an indefinite reproduction of their own forms.

Atoms and molecules in crystals are arranged in space lattices, geometrical configurations possessing definite elements of symmetry.‖ In virtue of this symmetry the lattice arrangement represents a state of minimum potential energy and is stable with respect to less ordered

† N. Semenov, *Chemical Kinetics and Chain Reactions*, Oxford, 1935; C. N. Hinshelwood, *Kinetics of Chemical Change*, Oxford, 1940.

‡ Cf. N. Rashevsky, *Mathematical Biophysics*, Chicago, 1938.

§ J. H. Northrop, *Crystalline Enzymes*, Columbia U.P., 1939; W. M. Stanley, *Phytopath.*, 1936, **26**, 305.

‖ For an account of this and all related matters see C. W. Bunn, *Chemical Crystallography*, Oxford, 1945.

configurations obtained by disturbing it. It is relatively difficult for the first few molecules of a substance to aggregate themselves with the correct orientations to form a minute crystal, because thermal agitation always tends to disperse them. But as the aggregate becomes larger it offers greater attraction to fresh molecules because it contains a number of attracting centres reinforcing one another. For this reason crystals grow most easily on existing nuclei, that is to say, they tend to increase by the reproduction of an existing pattern. The basis of all this is simply the tendency of matter to assume (in so far as thermal motions will allow) states of minimum potential energy.

This principle governs not merely the building of crystals from the corresponding liquid or vapour, but also the formation of new crystalline phases by chemical reaction. The dissociation of calcium carbonate always takes place preferentially at an interface of calcium oxide, where the activation energy is lower because the molecules or ions of the oxide are more stable when ordered in their own space lattice than they would be if randomly disseminated among the ions of calcium carbonate. Similarly the decomposition of arsenic hydride is catalysed by metallic arsenic. In general we may write

$$\text{catalyst} + \text{substrate} = \text{more catalyst} + \text{product}. \qquad (8)$$

The stable placing of molecules in the expansion of the existing lattice represents a decrease in free energy which can if necessary compensate accompanying increases in the simultaneous formation of other products.

It seems quite possible in principle that certain protein patterns in cells may extend themselves by removing suitable fragments from other molecules, leaving residues which can participate in further cell processes. The expansion of an existing pattern will be favoured in comparison with the creation of a new one.

11. Oriented overgrowths†

Two substances which are isomorphous can form mixed crystals, one type of molecule replacing the other more or less indiscriminately in the lattice. But there is a half-way house between this complete compatibility and the total lack of it. If there is a general similarity in the type and spacing of the atoms between one crystal face of one substance and another crystal face of a second, then one crystal

† See C. W. Bunn, loc. cit.

may be able to grow in a parallel orientation on the other. Such orientated overgrowths occur with sodium nitrate on calcite and urea on ammonium chloride.

What suggests itself here is that protein patterns may be able to guide the formation of further lines or sheets of protein molecules generally similar to but not absolutely identical with themselves. We shall have occasion to return to this idea later.†

12. Formation of macromolecules

Bacterial cells contain macromolecules such as proteins and polysaccharides, and these are built up from the very simplest of units such as ammonia and compounds containing not more than three carbon atoms. The condition that small molecules shall participate in the formation of macromolecules is that they should be polyfunctional.‡ A dibasic acid, for example, forms a semi-ester with a dihydric alcohol: there remain a carboxyl group and a hydroxyl group which can react with further molecules of alcohol and acid respectively. The process can be repeated until a polyester of enormous molecular weight is formed. In proteins, of course, the essential polyfunctional molecules are amino acids, which can give repeated —NHCO— linkages. Bifunctional molecules such as hydroxy-acids, amino acids, and olefines give macromolecules consisting essentially of chains. If further groups capable of condensation reactions are present, sheets and three dimensional arrays can be formed by the cross linking of chains. If the cross linking is frequent, the resulting structures will be insoluble and impermeable: if it is infrequent, easily permeable networks may be produced.

Many macromolecules are capable of crystallization, and not infrequently there occur mixtures of crystalline and amorphous material§ in which the same long chain molecules traverse both, as indicated in Fig. 5.

In principle, we can envisage regions possessing quite different kinds of crystalline order, spatially separated and yet held in definite relation by tie-molecules. This can come about if we have a long chain of condensed A, B units followed by a chain of B, C units. For example, the same giant molecule could be polyester at one end

† Cf. p. 180.

‡ W. H. Carothers, Collected Papers, in *High Polymers*, vol. i, Interscience, 1940.

§ Cf. C. W. Bunn, *Proc. Roy. Soc.*, A, 1942, **180**, 82; C. W. Bunn and T. C. Alcock, *Trans. Faraday Soc.*, 1945, **41**, 317; E. Hunter and W. G. Oakes, ibid., 1945, **41**, 49; H. C. Raine, R. B. Richards, and H. Ryder, ibid., 1945, **41**, 56.

and polyamide at the other. In such an event the two regions like (*a*) in Fig. 5 could be of quite different character. This would look something like the very first beginnings of an organized and differentiated structure, though separated by a vast distance from the organized structures of biology.

As has been observed, these huge molecules grow by the successive accretion of new units, the individual steps of the process not differ-

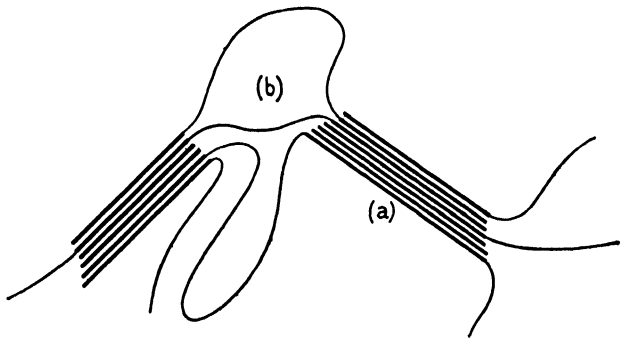


FIG. 5. Crystalline and amorphous regions in macromolecular substance.

ing in principle from those occurring in the reactions of substances of low molecular weight.†

13. Macromolecular constituents of bacterial cells

The most important high molecular weight constituents of bacterial (and other cells) are polysaccharides, proteins, and nucleic acids.‡ It is desired here to draw attention to a few specific facts only, which suggest ideas of far-reaching scope.

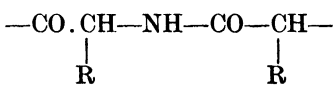
In the polypeptide chains of proteins a new kind of arrangement reveals itself, namely a folding of the chains into configurations which may possess a highly specific character. Various agencies, such as heat or adverse pH, bring about a process called denaturation, after which, according to the evidence of X-ray analysis, the regularity of the configurations is destroyed, and important properties of the protein are lost.

† H. Mark, 'High Polymeric Reactions', *High Polymers*, vol. iii, Interscience, 1941.

‡ W. T. Astbury, 'The Forms of Biological Molecules', in *Essays on Growth and Form*, Oxford, 1945.

Proteins have never been synthesized in the laboratory (by other than biological means), and the discovery of the specific chain-foldings suggests the reason why. Ordinary polycondensation reactions are not difficult to control: formally there is no special chemical intricacy in the reactions by which the parts of a protein might be put together. But it seems that the achievement of the characteristic foldings can only occur with the guidance of a pre-existing pattern, in other words *that the synthesis of a protein partakes of the nature both of a polycondensation reaction and of crystal growth.* Since the configurations are highly specific and capable of subtly graded variations, we begin to see (*a*) why living matter must always be derived from previous versions of the same, and (*b*) how subtle differences may exist and how finely differentiated changes may be impressed upon it.

It has been suggested† that the role of glycine in protein structure is a special one. In the chain



rotation will be considerably easier when R = H. The glycine residues, therefore, facilitate the folding of the chains, while other groups may determine the actual manner in which the folding occurs.

The nucleic acids are generally considered to play a key part in cell processes.‡ They consist of macromolecules built up from nucleotides, which are formed by condensation of a purine or pyrimidine base with a sugar (ribose or desoxyribose), the latter being itself linked to phosphoric acid. The nucleotides are connected together through the phosphoric acid molecules, and are superposed in a columnar manner, 'like a pile of plates in a tall plate-stand' as it is expressed by Astbury,§ who gives the diagram reproduced in Fig. 6. Astbury gives the distance between the successive layers of the column as 3·34 A and emphasizes that this is just equal to the distance between successive side chains in the direction of the main chain axis in the extended form of proteins. Once again, it is easier to envisage the reproduction of such structures when the synthetic processes are

† H. Neurath, *J.A.C.S.*, 1942, **65**, 2039.

‡ Cf. T. F. Dixon, *Nature*, 1945, **155**, 596; C. A. Schulman, *J. Heredity*, 1938, **29**, 414.

§ W. T. Astbury, loc. cit.

guided by an existing structure to which new elements aline themselves, than it is to picture its initial emergence from the chaos of molecular motions. The nucleus of an inorganic crystal is only formed with great difficulty compared with its expansion: the initial emergence from chaos of the master patterns of living material may possibly be of such transcendent improbability that it has occurred rarely—perhaps but once in the history of the world.

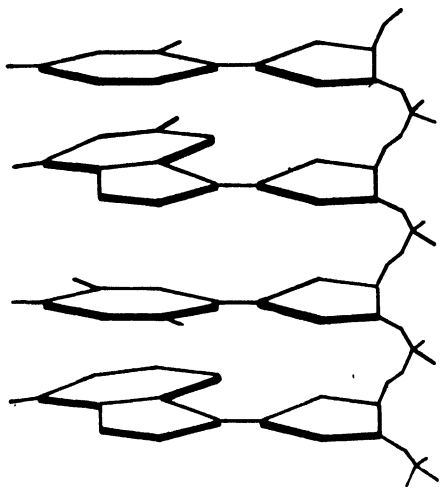


FIG. 6. Array of nucleotides.

14. The structure of bacteria

Bacteria possess nothing in the way of a definitely differentiated structure. The internal composition is not uniform, physically or chemically. A cell wall can be distinguished, and it is claimed by some writers that a nuclear apparatus† can be demonstrated by appropriate staining methods.‡ But the inhomogeneities observed are of a fine-grained and subtle kind. Thus G. Knaysi and S. Mudd,§ having examined *Staphylococcus aureus* and *Streptococcus pyogenes* by the electron microscope, came to the conclusion that no internal structure was detectable, and that the nuclear material must be in

† See Chapter X.

‡ C. F. Robinow, *Proc. Roy. Soc.*, B, 1942, **130**, 299; G. Piekarski, *Z. Bakt.*, 1939, **144**, 140.

§ *J. Bact.*, 1943, **45**, 349.

solution or in a state of very fine dispersion. *Staphylococcus flavocyaneus* contained certain granules which were thought to resemble nucleo-proteins in solubility. The general conclusion was that nuclear material of cells could be in different states according to the nature of the bacterium and its phase of development. Another electron microscope investigation, with some species of luminous bacteria, gave little evidence of internal structure.†

Some bacteria possess what are called flagella. These are minute processes protruding from the surface. They cannot, however, be regarded as separate organs.

The pneumococcus can surround itself with a so-called capsule, This is associated with a polysaccharide material, specific to the particular type of pneumococcus. It is stated that with careful technique the capsule can be revealed in electron microscope pictures as a definite structure surrounding the cell wall, which, in turn is demonstrable as a distinct structure bounding the inner protoplasm.‡

Bacteria do not contain chromosomes and the hereditary material appears to be dispersed, though not necessarily quite uniformly, throughout the cell. In biological terms there is the possibility, though not the certainty, that the gene may be identical with the soma.§ This offers some hope that the phenomena of heredity, variation, and adaptation may be understandable in terms of the physics and chemistry of the cell material as a whole. With most cells of plants and animals division occurs after a splitting of the nucleus and an elaborate rearrangement and division of the chromosomes themselves.‖ The question arises whether the greater simplicity of the reproduction process with bacteria is apparent or real. It is, on the one hand, conceivable that some invisible process of mitosis occurs, and, on the other hand, that the bacterial procedure is really simpler, and of a kind whose understanding will make the eventual interpretation of the more complex version easier. The latter seems to afford the better working hypothesis.¶

At any rate one is left with the impression that the internal structure of bacteria, and the mechanical processes accompanying their division are not so elaborate as to discourage all attempts to

† F. H. Johnson, N. Zworykin, and G. Warren, *J. Bact.*, 1943, **46**, 167.
‡ S. Mudd, F. Heinmets, and T. F. Anderson, ibid., 1943, **46**, 205.
§ The idea of localized genes has recently gained very much in favour.
‖ D'Arcy Thompson, *Growth and Form*, Cambridge, 1942, Chapter IV.
¶ Cf. Chapter X.

understand the physical chemistry of the macromolecular and other reactions which occur in them.

15. Some general remarks on cell mechanisms

At this stage it may be useful to consider whether there seems, in principle, to be any prospect of understanding the chemical kinetics of bacterial growth and behaviour in terms of facts and principles similar to those which have just been recapitulated. There is no question of establishing hypotheses, but merely of forming an opinion in a preliminary way of the kind of hypotheses which might be possible.

Having regard, on the one hand, to the absence of gross structural differentiations in bacteria and, on the other hand, to the varied constitutions and foldings of proteins and to the spatial arrangements of nucleotides, one has the impression that there is no clear necessity to go outside macromolecular chemistry to understand the actual architectural units which make up the cell.

It seems highly probable that the reproduction of the structure is explicable in terms of the gain of stability occurring when fresh elements fit into their place in the expansion of an already ordered array. Into the source of the initial ordering we are not called upon to inquire.

The chemical nature of the synthetic steps constitutes a problem difficult in detail but attackable in principle. The cell possesses numerous enzymes, and each part of its substance is fully reproduced during growth, and if a steady supply of raw material is maintained and toxic products removed, this reproduction continues indefinitely according to an exponential law. If m_0 is the initial mass of any kind of cell substance and m the amount at time t, then $dm/dt = km$ or $m = m_0 e^{kt}$. This is the fundamental law of growth, and is, of course, based upon experiment. Moreover, in a constant medium the various enzymes† and other cell substances are formed in a constant ratio, so that they must all obey the same law of autosynthesis. Thus an important equation of growth processes must be:

$$\textit{enzyme} + \textit{substrate} = \textit{more enzyme} + \textit{products (possibly utilizable in further syntheses)}. \quad (9)$$

† It will be convenient in future discussions to call any part of the cell texture where chemical reactions occur an 'enzyme'. What are usually called co-enzymes will, according to circumstances, be treated either as parts of the enzyme structure, or as reactants in one or other of the series of linked processes.

This sort of law is intelligible by analogy with that expressed in equation (8).

Different regions of the cell possess different functions, probably determined by the molecular pattern and by the specific active groups prevailing locally. These various departments of the cell, according to one quite possible picture, would not so much resemble the separate rooms of a house as the counters in a large multiple store. They must be held in a kind of spatial organization, and this could be imagined to occur through the intervention of polyfunctional tie-molecules linking the various regions. The whole lay-out will differ in density and permeability from point to point, some parts constituting practically crystalline protein, others being left open for permeation by aqueous solution of diffusible materials.

The great variety of chemical reactions which can be brought about is most easily understood if the enzymatic processes are thought of as resolved into sequences of rather simple steps (for example, removal of hydrogen atoms, addition of oxygen atoms and so on), which can be combined and permuted in all sorts of ways, so that the initial raw materials are operated on by a whole succession of enzymes. Intermediate products, many of which must be very active and labile, must diffuse from one region of enzymatic activity to another. The concentrations in which the intermediates reach the next enzyme of a sequence depend upon the spatial distribution of matter in the cell—and this applies whether or not they are labile substances. On their way from one processing department to the next they may either suffer partial decay, if unstable, or, if stable, be exposed to alternative fates such as loss by diffusion.

According to this general picture, the activity of the cell consists in a sequence of reactions with relative speeds determined by the spatial relations as well as by the specific chemical properties of the substances involved. In a steady state, definite concentration gradients will be established between one enzyme region and the next. This is simply a rather elaborate form of what is referred to in § 7, but it gives us a glimpse of how a spatio-temporal organization could arise. The fundamental geometry of the macromolecular systems will determine the spatial relations of the enzyme regions, the chemical character of those regions the reactions which occur there, and the different concentration gradients help to control the sequence and relative rates. One can only speculate—and even that

imperfectly– as to the centre which determines the whole macromolecular arrangement, and upon such problems as the part played by the stereo-chemistry of the complexes formed by the various metal atoms (e.g. iron) which have considerable importance in all life.

We now come to some crucial questions: those, namely, of how the autosynthetic reactions are initiated, how they are propagated with such ease while the cell remains alive, how they may be interrupted for considerable periods during the non-proliferating periods of the cell's existence and yet renewed again with the appropriate change of conditions, and finally what happens when the cell dies. Once again, we do not seek answers to these questions here, but ask merely whether physico-chemical answers seem in principle possible. Free radical mechanisms provide one suggestive possibility. If a free radical reacts with a saturated molecule a new free radical is produced. In the ordinary chemical processes of the laboratory such free radicals must continue to be kept in play or they are lost by various recombination processes unhelpful to the main reaction. With the elaborate structural organization of the cell one can imagine their preservation for long periods: they do not combine because they are fixed in space, being simply unfinished ends of a more or less rigid structure. In such a way they could remain mobilized for use for considerable periods. If the structure decays (e.g. by denaturation of protein) or the radicals are used up by foreign bodies not utilizable in growth, the cell dies. From this point of view the macromolecular structure of the living cell might be regarded as an extremely elaborate and finely adjusted support for active radicals, and the death of the cell would represent on a higher plane what on a lower plane occurs in the sintering of a catalyst, or in the disappearance of the free radicals after the light is turned off in a photochemical reaction.

In this connexion it is worth considering that, even when not growing, cells frequently engage in respiratory processes involving consumption of oxygen, and that oxidation processes in general are fruitful sources from which radicals can be formed or renewed.

Whether or not actual cells resemble the system pictured here cannot be said: but the converse can be affirmed, namely that such a system would show some of the properties required.

On the whole, then, it would seem that the possibility of an autosynthetic structure is—given the right initial conditions—con-

ceivable in not wholly unfamiliar terms. As such a structure expands by utilizing raw materials supplied from outside, the scale effect must come into play in the manner indicated in § 9. No steady expansion according to the [illegible] autosynthetic growth law will be possible unless in some way an approximately constant set of conditions can be maintained, and this involves a preservation of an approximately constant surface-volume relation. The required relation is in fact preserved by the division of the cell at more or less regular intervals, and we have to inquire whether any analogies exist which help in the understanding of this. The two most obvious do not seem at first sight to take us very far. One is the fact that crystals which have grown to a large size, although having the appearance of a single geometrical figure, are nearly always found on inspection to consist of a mosaic of very many small crystals. But here there is no question of a two-way diffusion process in the system. The second analogy is that of a column of liquid breaking up into small drops under the influence of surface tension. This is not very helpful either, as will be seen in a later chapter. It would seem rather that as growth proceeds the changing concentration gradients alter the ratio of the chemical processes, and that the changed chemical balance in the cell sooner or later provokes a reorganization. The precise mode of action is mysterious and will not be discussed in detail here. One may, however, remark that certain concentrations could increase steadily as the area-volume ratio dropped, until at a given point a coagulation or precipitation occurred, and that such an event might serve as the origin for fresh nuclear material (or indeed for the formation of fresh kinds of substance such as cell wall). The foregoing remark is not a hypothesis about division, but is intended to call attention to the fact that, in terms of the scale effect, the cause of division is unknown but not necessarily unknowable.

16. Matters to be dealt with in subsequent chapters

To attempt to correct or to fill in the picture vaguely indicated in the last section would be an ambitious task, which would go very much beyond what is contemplated in the following pages. At the same time, the physico-chemical outlines which have been sketched constitute the framework in which the shorter range studies which follow are to be set.

What will be sought will be to discuss various phenomena connected with the growth, activity, and adaptation of bacteria, and with the action of various chemical substances on them, in the light of physico-chemical principles. What, with good fortune, may appear is not that the enormous intricacies can be unravelled at present, but rather that, in spite of these, certain intelligible relationships may be discerned.

II

THE GROWTH CYCLE OF BACTERIA

1. Preliminary information about bacteria

SINCE we are to be concerned with what are essentially physico-chemical experiments on biological material it will be convenient at the outset briefly to enumerate those facts about bacteria themselves which will be needed by chemists who have not previously acquainted themselves with the terminology of bacteriology.†

Bacteria, as already stated, are unicellular organisms which multiply by elongation and binary fission. They are isolated from air, from the soil, from animal tissues and fluids both in health and disease, and from other varied and sometimes improbable sounding locations. Unfortunately bacteriology has rather frequently been the mere handmaiden of medicine, a fact which has tended to give undue prominence to pathogenic organisms. But invasion of animal tissues is only one of very many activities, which include fixation of nitrogen, formation of methane, and even the consumption of elementary sulphur to form sulphuric acid. Nowadays collections of pure strains exist, for example, the National Collection of Type Cultures, from which the starting material for investigations can conveniently be obtained.

The size and shape of bacteria are capable of variation between fairly wide limits. Generally speaking there are two main divisions, cocci, which are spheroidal in shape and have diameters in the region of $1\,\mu$, and rod-shaped cells, the length of which is usually between 1 and $10\,\mu$, and the breadth from $0{\cdot}2$ to $1\,\mu$. Under special conditions much longer filaments may be formed, and occasionally Y-shaped cells‡ are produced (which can divide in such a way as to produce new cells at each arm of the Y). The rods are characteristic of the genera *Bacterium*, *Bacillus*, and others.

There is a more or less generally accepted system of classification of bacteria, which, however, is not of special interest for the present purpose, being based, from practical necessity, upon somewhat mixed categories. In one group the species are distinguished by their specific

† For biological and biochemical information see A. D. Gardner, *Bacteriology for Medical Students and Practitioners*; W. W. S. Topley and G. S. Wilson, *Principles of Bacteriology and Immunity*; M. Stephenson, *Bacterial Metabolism*.

‡ A. D. Gardner, *J. Path. Bact.*, 1925, **27**, 189.

fermentation reactions with sugars, in another by the action upon blood, and so on. All that will be necessary here will be to mention some of the important genera and species. Among cocci, the two main classes are *Staphylococcus* and *Streptococcus*. In the former the spheroidal cells tend to remain bunched together in grape-like clusters, whereas in the latter they tend to remain adhering to one another in long chains. Both groups are associated with febrile and pus-producing infections, and the sub-divisions on the whole are based upon criteria which are principally of concern to pathology. The genus *Bacterium* is an important one containing several common species. *Bacterium coli* is the common inhabitant of the intestine, *Bacterium lactis aerogenes* (sometimes *Aerobacter aerogenes*) is a closely related species obtainable from soil, *Bacterium typhosum*, *Bacterium paratyphosum A*, and *Bacterium paratyphosum B* are respectively responsible for typhoid and the two commoner forms of paratyphoid. There are also various forms of *Bacterium dysenteriae*. All these constitute a rather well-defined group, the subdivisions of which rest upon biochemical reactions such as the specific fermentations of sugars.

Another large genus of rod-like organisms is that of *Bacillus*, which includes many species pathogenic and non-pathogenic. Typical members are *Bacillus anthracis*, and *Bacillus subtilis*, the latter frequent in atmospheric dust. The outstanding characteristic of the group is their ability to assume an alternative morphological modification, considerably more resistant to heat and chemical agencies and known as a spore. Spores are normally more nearly spherical than the bacilli from which they are derived, and more refractile. They also have different staining properties. They represent a resting form of the cell and when placed in suitable nutrient media are re-transformed into bacilli, a process referred to as germination. Germination must precede division. Another spore-forming genus is termed *Clostridium*.

Two other genera only will be mentioned here: *Corynebacterium*, of which *C. diphtheriae* is a typical member and which is characterized by having a rod-like form with slightly swollen, or 'club-like', ends: and *Mycobacterium*, characterized by the presence of a waxy constituent and known from certain consequential staining properties as 'acid-fast'. The bacteria of tuberculosis belong to this class.

Bacteria are divided into two classes known as Gram positive and Gram negative respectively, according to their behaviour in the Gram staining test. The cells are treated with methyl violet and then with dilute iodine solution. After this they are washed with alcohol or acetone for a standard time. With the one class the stain is retained and with the other it is lost. The test depends upon some physico-chemical characteristic of the surface of the cell. Although the behaviour of some bacteria is rather indefinite the Gram test on the whole separates the genera into two main groups. That the division has a real significance is suggested by various facts, for example, that Gram-positive bacteria are affected by penicillin while Gram-negative bacteria are not. Of the genera which were (somewhat arbitrarily) selected for mention above, *Streptococcus*, *Staphylococcus*, *Mycobacterium*, *Corynebacterium*, and *Bacillus* are Gram-positive while *Bacterium* is Gram-negative. Plenty of other Gram-negative genera are, however, known.

Bacteria are very variable, and although the main species characteristics usually remain fixed, a great deal of doubt and controversy surrounds the question of detailed subdivisions. This matter will not, however, be pursued here, since it will be discussed in some detail in connexion with adaptation and variation.

Bacteria, of course, assume the temperature of their surroundings. They survive cooling to very low temperatures, but are usually killed by heating to about 55–65°. Some species, called thermophilic, not only survive, but flourish at higher temperatures than these. Spores usually survive even prolonged heating at 100°. For many bacterial species the temperature of optimum growth is between 30° and 40°.

2. Cultivation

Only enough will be said here about the cultivation of bacteria to make intelligible the references to experimental work in later parts of the book. There is no necessity for the purpose in view to describe matters of technique such as aseptic working, all-important as such details are from the experimental point of view.

Bacteria may be cultivated on solid or in liquid media. The former consist usually of a jelly (such as agar agar or gelatine) to which appropriate nutrient substances have been added. A suspension containing the bacteria at a suitable dilution is smeared over the surface (or sometimes inserted by puncture, or mixed before setting

of the jelly) and the preparation, usually in a shallow covered glass dish, is incubated for a day or more at a suitable temperature. *Colonies* of bacteria grow, each from a single cell. If the original dilution was suitably judged, these colonies are well separated from one another. They are more or less circular in shape, and quite often about 1 mm. in diameter (though the size varies a good deal from case to case). According to their actual appearance and texture colonies are classified as *rough* (*R*) and *smooth* (*S*), the former having wavy edges and a crinkly surface, the latter being even and glossy. Many bacteria are capable of giving both types, a phenomenon to which further reference will be made at a later stage.

The cultivation of colonies is the normal means of isolating pure strains of bacteria. Since each colony grows from one cell, a mixture of bacteria can be separated by selecting individual colonies. (Another technique consists in the direct manipulation of single cells under the microscope.)

Many liquid media are used for growing bacteria. A common one is bouillon or broth, prepared from meat, but, for the kind of experimental work with which we shall be chiefly concerned, synthetic media consisting of sugars, amino acids, certain salts, and, in some cases, specific growth factors are more satisfactory.

A small quantity of a parent culture, the *inoculum*, is transferred to the culture medium. As fresh growth proceeds the medium becomes turbid, and the course of the proliferation can be followed either by turbidimetric measurements, or by counting samples under the microscope. For the latter purpose a small chamber of accurately known depth and provided with fine rulings of known spacing can be used. Turbidity is perceptible with about ten million cells per c.c.: and with some strains of bacteria the growth goes on till the number per c.c. exceeds a thousand million.

For many purposes it is expedient to maintain the strain by *serial subculture*. When fully grown, a given fraction (say 1/100, 1/1,000, or 1/10,000) of a given culture is used as inoculum for a fresh supply of the original medium. This process will be referred to rather frequently.

It is advantageous to bubble a gentle stream of air through many cultures during growth. A class of bacteria known as anaerobes, on the other hand, must be grown in almost complete absence of oxygen. Bacterial enzymes do not operate solely when cells are growing.

The culture may be centrifuged and the cells separated from the growth medium. They may then be suspended in appropriate solutions in which chemical reactions, such as reduction, deamination, hydrogen peroxide decomposition, and so on, are caused to take place by the bacterial enzymes.†

3. Raw materials required for bacterial growth

Sources of carbon and nitrogen are obviously needed. Phosphorus and sulphur must be supplied, but inorganic phosphate and sulphate are often sufficient. Traces of iron and magnesium are usually required as well as sodium, potassium, and calcium. Some bacteria require gaseous oxygen and most, if not all, require a small concentration of carbon dioxide.

The sequence of cell reactions can begin with very simple chemical substances, since some bacteria will grow readily in media which contain ammonium ion or nitrate ion as the sole source of nitrogen, and a comparatively simple compound such as glycerol as the sole source of carbon. From such raw materials all the complex and varied products can be built up by what must be a very versatile and adaptable system of transformations.

With many bacteria, however, more elaborate molecules must be supplied ready-made, or growth is impossible. The demands in this respect are continuously graded between the so-called 'exacting' and the 'non-exacting' strains.‡

Certain bacteria refuse to grow except in quite specialized environments: *Mycobacterium leprae*, for example, is stated never to have been cultivated artificially at all—though this is rather exceptional. More frequently definite growth factors or combinations of growth factors have to be provided. For example, most of the pathogenic streptococci need ready-made glutamine.§ In the absence of air *Staphylococcus aureus* in an artificial medium was found to need uracil and according to Richardson|| none of twenty-one somewhat similar compounds would serve instead. *Staphylococcus aureus* has also to be provided with a potential source of the —SH group,¶ provided

† J. H. Quastel, *J. Hyg.*, 1928, **28**, 139.

‡ See especially B. C. J. G. Knight, *Bacterial Nutrition*, H.M. Stationery Office, 1936.

§ H. McIlwain, P. Fildes, G. P. Gladstone, and B. C. J. G. Knight, *Biochem. J.*, 1939, **33**, 223.

|| G. M. Richardson, ibid., 1936, **30**, 2184.

¶ P. Fildes and G. M. Richardson, *Brit. J. Exp. Path.*, 1937, **18**, 292.

in an organic compound. Many examples of this sort of demand are known. In addition to glutamine and uracil, one might mention nicotinic acid, thiamin,† tryptophan,‡ and various amino acids or combinations of amino acids.†

A natural hypothesis is that compounds similar to these various growth factors are intermediates in the chain of processes occurring in cells which can start with simpler materials. This receives support from the fact that many bacteria as originally isolated (for example, from animal tissues in which they have been parasitic) require definite organic compounds for growth, but can be trained to dispense with them. The training is effected by serial subculture with gradual reduction to zero of the supply of the compound in question. For example, *Bact. typhosum* normally requires tryptophan, which is an essential constituent of protein, but can be trained to build it up, first from indole and then from ammonia.§ This suggests strongly that bacteria which utilize ammonia to form the tryptophan units of their substance do so by way of indole.

In the light of such observations on the gradations in the series of growth requisites passed through during training, it would seem that the essential difference between the exacting and the non-exacting types of bacteria is merely the precise point in the series of reactions at which operations begin. In the exacting species the necessary enzymes for the earlier stages of the sequence are either absent or are inactive until they have been developed by the training process. When cultivated in a highly specific environment where complex organic substances important to cell life are freely provided, the enzymes necessary for the simpler initial stages of synthesis pass out of use. In this way pathogenic (i.e. parasitic) bacteria usually become exacting.

The intermediates added as ready-made growth factors are not necessarily used in the form in which they are supplied: they may rather be the source of active fragments common to more than one possible reaction. When *Staphylococcus aureus* is trained to grow without added alanine, it becomes able to dispense also with valine, leucine, and histidine, which it normally demands.‖ This suggests

† B. C. J. G. Knight, *Bacterial Nutrition*, 1936.

‡ P. Fildes, G. P. Gladstone, and B. C. J. G. Knight, *Brit. J. Exp. Path.*, 1933, **14**, 189.

§ P. Fildes and B. C. J. G. Knight, ibid., 1933, **14**, 343; P. Fildes, ibid., 1940, **21**, 67.

‖ G. P. Gladstone, ibid., 1937, **18**, 322.

that the mechanisms which are mobilized on training deal not specifically with the individual amino acids but with active fragments common to them all.

The over-all picture is that of a sequence of linked reactions which begins with the utilization of such simple compounds as carbon dioxide and ammonia, and builds up successively more complex molecules. Some of these are diffusible and pass from one region of enzymatic activity to the next, there to be used in the further stages of the sequence. At each stage macromolecular polycondensation reactions must occur since on growth the actual substance of each part of the cell is reproduced. The general scheme must include that given by equation (9) of Chapter I.

The chemical functions of the various regions of enzymatic activity may well be relatively simple and the changes occurring at some of them may well yield fragments of great reactiveness such as free radicals. If there are a large number of such regions, then the permutations and combinations possible are very great and the synthetic and transformative power of the cell will be as varied as it is intense. It is helpful to think of the chemical operation of the cell less as the piecing together of a jig-saw cut into large fragments which will fit together in one way only, than as the formation of a mosaic from simple units which can be combined in innumerable ways. There will be occasion later to return to this analogy, and to express it in a somewhat different form.

4. Phases of growth

According to what has been said, a growing cell constitutes a manufactory with numerous departments, the conveyor belt leading from one to another being represented simply by the appropriate concentration gradients. Raw materials are broken down and built up into fresh forms in a series of stages. Moreover, since the material of the cell is constantly increasing in amount, and since new cells continually formed by division are nearly identical with the original ones, every single part of the machinery must devote some of its effort to making more of the same kind. If all the raw materials are supplied from a constant environment, a steady state must be established in which all parts of the cell material expand at rates such that their relative proportions remain unchanged. As has been explained (p. 15) the scale effect would interfere with the maintenance

of a steady state were it not for the phenomenon of cell division, which enables it to be preserved. With regular division, each cell can go on growing at the same rate as its parent. Hence the total number increases with time in geometrical progression according to the law

$$n = n_0 e^{kt},$$

where n_0 is the original number and n the number at time t, k being a constant. This law is in fact rather closely followed (with understandable deviations which will be discussed later) over quite wide ranges of growth. The steady state during which the above law is followed is known as the *logarithmic phase* of growth.

In practice, cells are seldom grown in a constant environment. Usually they are inoculated into a given volume of medium and allowed to grow, either until an essential raw material has been exhausted, or until the metabolic products which they themselves form inhibit further multiplication. The total number of cells then remains constant. The culture is now in the *stationary phase*. The total *stationary population* is to be distinguished from the number of living cells (viable population), which begins after a time to decline.

During the stationary phase certain changes must occur in the living cells themselves. In the first place, the activities of the various enzymes decline. This can be shown by direct experiments on washed cells.† In the second place, various intermediates of low molecular weight will be lost from the cell by diffusion. The first effect changes the nature of the actual sites of reaction, while the second completely alters the concentrations and concentration gradients which had been established in the steady state. Both combine to prevent the story from being taken up just where it was left off, even if the cells are transferred to fresh medium. The result is that, before steady growth can be re-established, there is in general a time interval known as the *lag phase*. During this the various intermediates are accumulated once more in the requisite concentrations, and the activities of the enzymes are restored. The latter process may involve a certain amount of reconstruction of material, in the likely event that some denaturation of the protein occurred during the stationary phase. This seems quite probable, since there is a continuous decline in the

† E. F. Gale, *Biochem. J.*, 1940, **34**, 392, 846; W. R. Wooldridge and V. Glass, ibid., 1937, **31**, 526; D. D. Woods and A. R. Trim, ibid., 1942, **36**, 501.

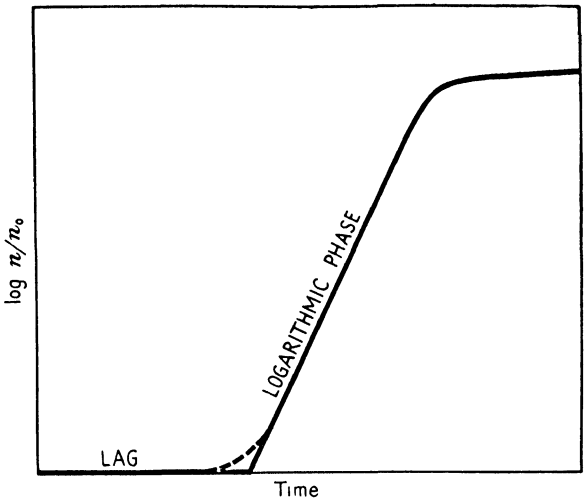


FIG. 7. Bacterial growth cycle: total numbers.

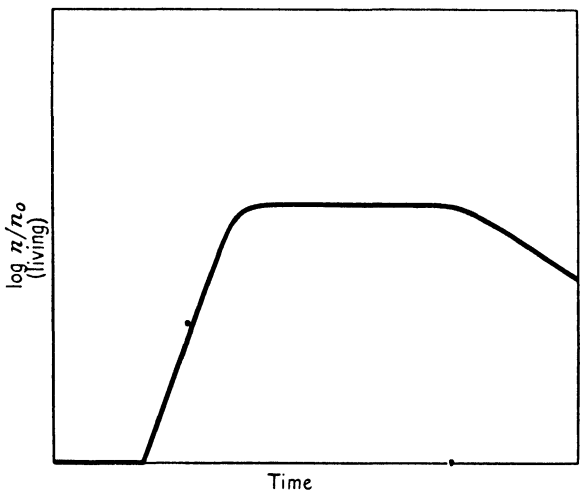


FIG. 8. Bacterial growth cycle: numbers of living cells.

various functions ending with the actual death of the cell if the stationary phase is unduly prolonged.

The expected sequence of events is indeed observed when cells are inoculated into fresh medium. A lag, the length of which is a

function of the previous history, is followed by a period of logarithmic multiplication. Growth then slows up and ceases. The relation between the total number of cells and the time from inoculation is

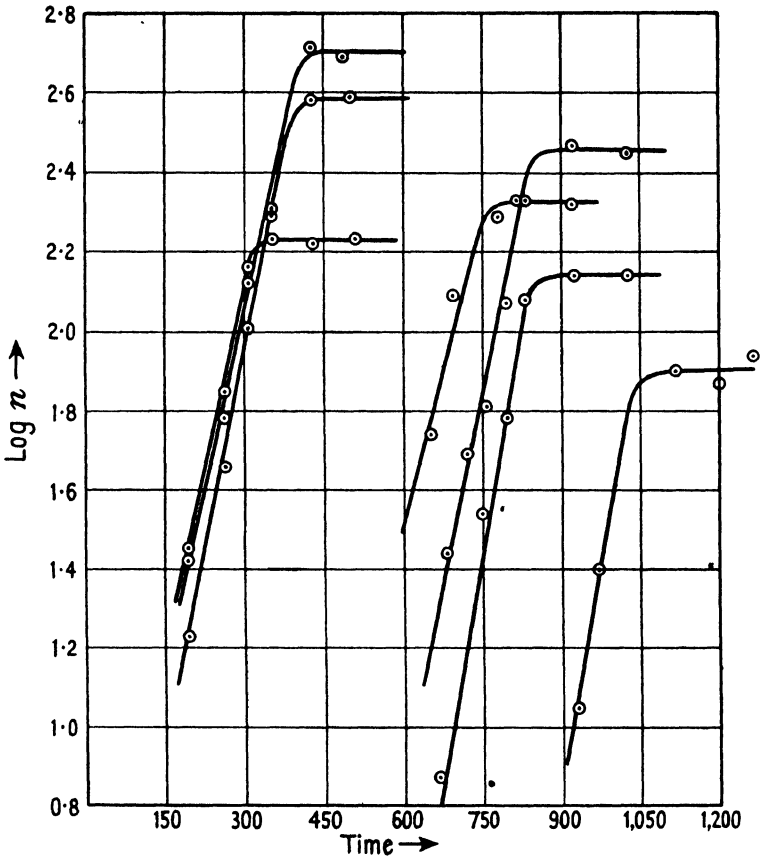


FIG. 9. Experimental growth curves for *Bact. lactis aerogenes*.

shown by Fig. 7. The relation between the number actually living and the time is somewhat different and is shown by Fig. 8. Fig. 9 gives some actual growth curves for a strain of *Bact. lactis aerogenes* grown in an artificial medium at various concentrations which support correspondingly different values of the stationary population.

If cells have been repeatedly cultured in a given medium and are transferred to one of a different composition, the proportions of the

enzymes which they contain may be far removed from that corresponding to optimum growth. The various reactions of the sequence involved in reproduction, may be completely out of balance and a steadily maintained logarithmic increase may not occur until several subcultures have been made. Fig. 10 shows some irregular growth

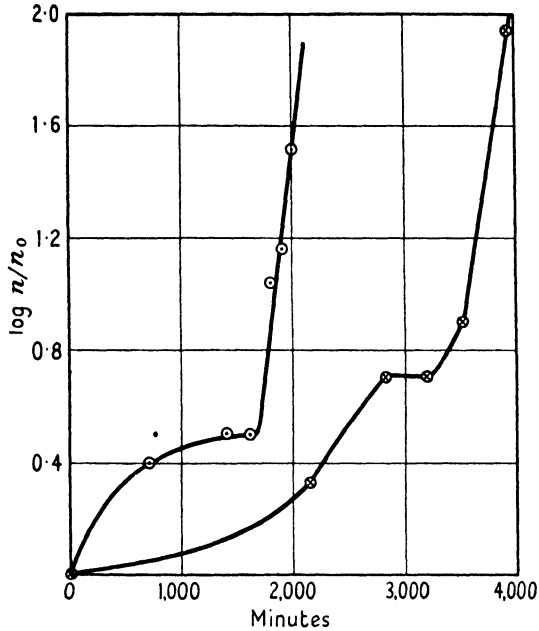


FIG. 10. Irregular growth curves of freshly transferred *Bact. coli mutabile.*

curves obtained with *Bact. coli mutabile* just transferred to a synthetic medium after cultivation in broth. Fig. 11, on the other hand, shows the almost ideally logarithmic form of curve attained after a considerable number of subcultures in the artificial medium (consisting of ammonium sulphate, lactose, and salts).

5. Experimental measurement of lag and growth rate

The law of growth is $$dn/dt = kn,$$

where n is the number of cells present at time t, and k is a constant. The limitations of the law, and the corrections which should be applied in certain circumstances, will be discussed later.

Integration gives: $\ln(n/n_0) = k(t-L)$,

where n_0 is the original number of cells and L is the time from the beginning of the observations (usually the moment of inoculation) at which growth according to logarithmic law begins. L is the lag.

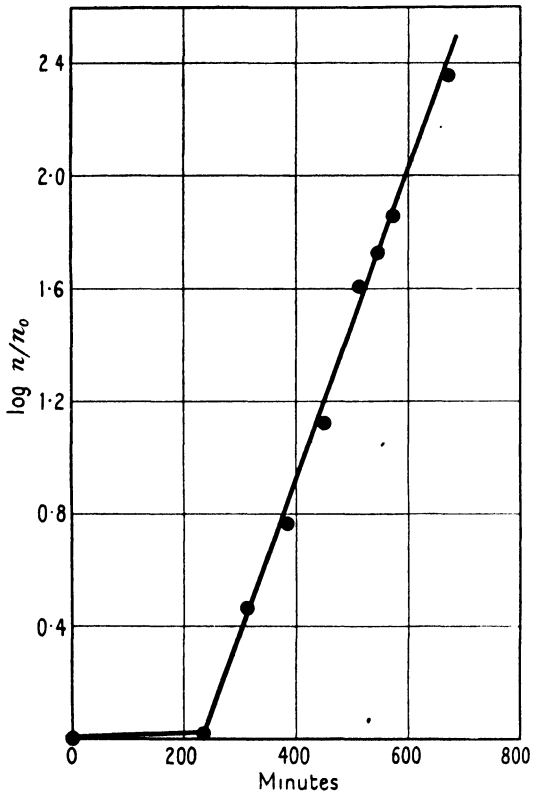


FIG. 11. Logarithmic growth of adapted *Bact. coli mutabile*.

k determines the growth rate. A convenient quantity by which to characterize the growth is the *mean generation time*, that is, the time required for the number of cells to double during the logarithmic phase. Representing it by T we have the equation

$$\ln 2 = k(t_1-t_2) = kT,$$

whence

$$T = \ln 2/k = 0{\cdot}693/k.$$

The mean generation time is best found from a plot of $\ln n$ against time.

From such a plot by extrapolation to $n = n_0$, the value of L may be read off. (Alternatively one plots $\ln(n/n_0)$ against time and extrapolates to zero.)

The transition from the lag phase to the logarithmic phase is not always sharp: it may be gradual as shown by the dotted line in Fig. 7. In such a case it is still convenient to define the lag as the intercept of the truly logarithmic part of the curve on the time axis.

To characterize the behaviour of a bacterial culture it is desirable to determine L, k, and the total population reached by the time growth ceases. Instead of k it is equally convenient to record the mean generation time.

III

THE PHASES OF THE GROWTH CYCLE

THE LAG PHASE†

1. General

THE foregoing discussion shows that, from the nature of the chemical processes in the cell, one must in general expect an induction period or lag during which the steady state corresponding to logarithmic growth is being established.

During the early stages of the lag phase there is no apparent increase in cell substance. As the end of it approaches, there is an increase in cell volume and this is usually heralded by an increased production of metabolites such as carbon dioxide.‡ There must naturally be two types of reaction in a cell, that which is not directly linked with the autosynthesis of cell substance (type *A*) and that (type *B*) which is so linked. Some of the essential intermediates will be built up by type *A* reactions and these doubtless predominate during the earlier stages of the lag. Type *B* will come into play more and more as the end of the lag is reached and will be responsible for the observed increase in cell size.

The lag is usually shorter in media, such as meat extract, which contain a varied stock of ready-synthesized compounds than in the simpler artificial media in which more preliminary stages have to be gone through.

2. Lag and concentration of medium constituents

The information on this subject is not very extensive. Bacteriologists have usually been content for their own purposes to record qualitative observations about the total growth after a standard time, a method which leaves one in doubt whether lag, growth rate, or total population have been the major factors involved. These three factors may vary in complete independence.

When artificial media are thoroughly freed from carbon dioxide by a stream of purified air, the growth of several typical species of bacteria is delayed indefinitely.§ In broth, the cells may cause an

† The work of earlier writers, among whom Penfold must be specially mentioned, is summarized in bacteriological text-books. A summary of more recent investigations is given by Winslow and Walker, *Bact. Rev.*, 1939, **3**, 147.

‡ G. Mooney and C.-E. A. Winslow, *J. Bact.*, 1935, **30**, 427; E. Huntingdon and C.-E. A. Winslow, ibid., 1937, **33**, 123.

§ G. P. Gladstone, P. Fildes, and G. M. Richardson, *Brit. J. Exp. Path.*, 1935, **16**, 335.

active enough fermentation to produce carbon dioxide more rapidly than it can be carried away by the air-stream. The carbon dioxide affects not only the lag, but also the growth rate, as has been found for *Bact. lactis aerogenes*† and for *pneumococcus*.‡ With the former the optimum amount of carbon dioxide corresponded to about 0·15

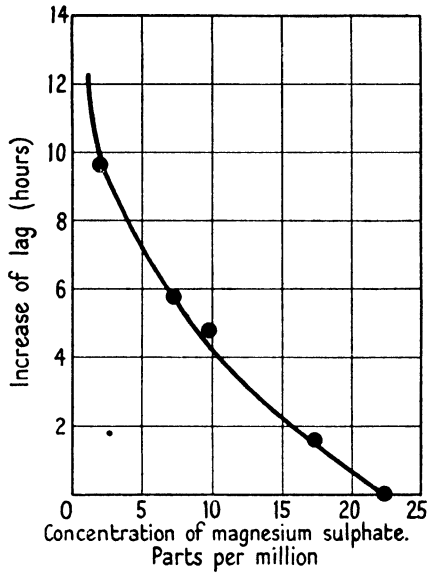


FIG. 12. Lag increase on reduction of magnesium concentration.

per cent. in the gas phase, which, under the conditions of working, corresponded to about 4×10^{-8} gram mol. per c.c.† Carbonate and bicarbonate ions will not replace carbon dioxide itself.§

In an artificial medium containing glucose and a phosphate buffer the lag of *Bact. lactis aerogenes* is indefinitely prolonged as the concentration of magnesium ions is reduced towards zero.‖ Biochemical evidence shows that magnesium is a phosphatase activator,¶ so that the undue slowing down of a phosphorylation reaction can evidently cause the observed prolongation of the lag. Fig. 12 shows

† S. Dagley and C. N. Hinshelwood, *J. Chem. Soc.*, 1938, 1936.
‡ W. Kempner and C. Schlayer, *J. Bact.*, 1942, **43**, 387.
§ G. P. Gladstone, P. Fildes, and G. M. Richardson, *Brit. J. Exp. Path.*, 1935, **16**, 335.
‖ R. M. Lodge and C. N. Hinshelwood, *J. Chem. Soc.*, 1939, 1692.
¶ L. B. Pett and A. M. Wynne, *Biochem. J.*, 1933, **27**, 1660; E. Bamann and W. Salzer, *Ber.*, 1937, **70**, 1263.

the manner in which the lag increases as the magnesium concentration falls.

With some strains of *Bact. lactis aerogenes* the lag increases somewhat with increase in the glucose concentration (of an ammonium sulphate, glucose, phosphate buffer medium) from 4 to 40 grams/litre, but with others it remains almost constant over this sort of range. Change in the ammonium sulphate concentration of the same medium from 0·2 to 5 grams/litre has also little effect. This is shown in Fig. 16*c*.

These results are hardly surprising, since the concentrations are large, even at the lower ends of their respective ranges. Experiments with very low concentrations are not easy, since, in the range where a significant effect on the lag might well appear, there would be too little total carbon or nitrogen in the medium to support appreciable growth. From the numbers quoted above in connexion with the carbon dioxide requirements it can be seen how small are the concentrations at which optimum effects are reached in respect of lags or growth rates. But the matter is quite different in respect of total population, the support of which demands quite large supplies of material even though the concentration at which it is furnished is not important.

More surprisingly the influence of pH on the lag phase of *Bact. lactis aerogenes* in an artificial phosphate-glucose medium is almost negligible, over a range in which numerous enzymes show very wide changes in activity. The contrast is illustrated by Fig. 13 and Fig. 14 (with which also Fig. 16*d* may be compared). The precise significance of this insensitiveness of the lag to the hydrion concentration is not clear, but it seems evident, at any rate, that a rather specialized explanation is required. It would be of interest to know more about the variation with other organisms and with other types of media.

The lag is largely and specifically influenced by various drugs, and this influence is markedly subject to adaptive changes. This subject will be considered at length in a subsequent chapter.

3. Lag and age of cells

Lag depends upon the age of cells.† The quantitative study of the lag phase fully confirms the idea that bacteria which can utilize the simplest sources of carbon and nitrogen build up their substance

† W. J. Penfold, *J. Hyg.*, 1914, **14**, 215; R. M. Stern and W. C. Frazier, *J. Bact.*, 1941, **42**, 479.

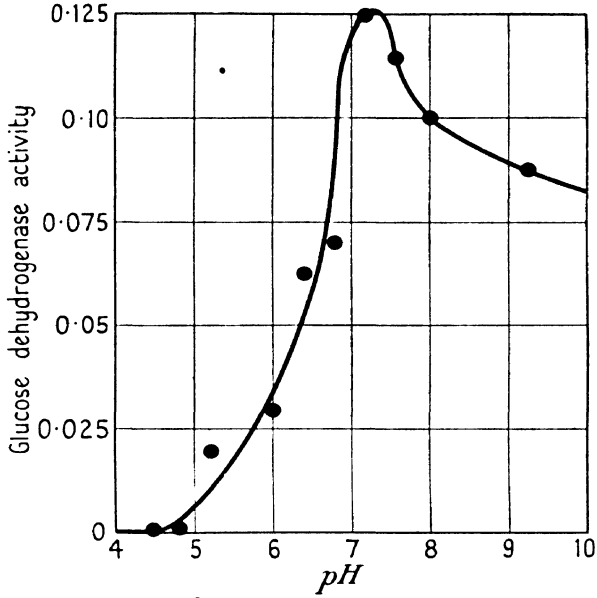


FIG. 13. Variation of glucose-dehydrogenease activity of *Bact. lactis aerogenes* with pH.

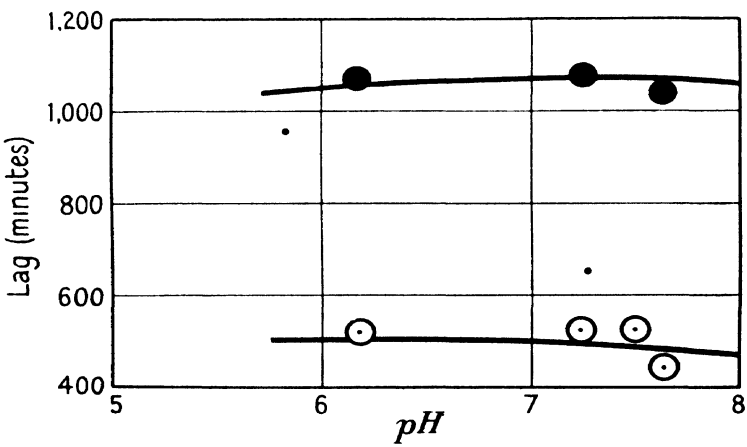


FIG. 14. Variation of lag with pH.

by way of intermediates which can sometimes diffuse from the cell into the medium, and which, therefore, must certainly be able to pass from one internal department of the cell to another in the manner suggested by the conveyor-belt analogy.

We shall begin with the consideration of some quantitative work on *Bact. lactis aerogenes*.† This bacterium grows well in a medium consisting of a phosphate buffer of pH 7·1, glucose, a small amount of magnesium, and, as source of nitrogen, either ammonium sulphate

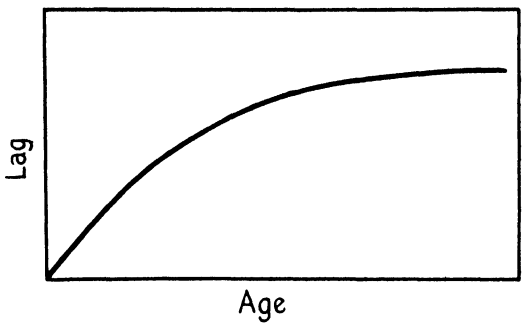


FIG. 15. Lag-age relation in amino acid medium.

or an amino acid type of compound such as asparagine. Cells may be transferred from a growing culture to a fresh supply of the same medium and the length of the lag may be determined. It proves to be a definite and characteristic function of the age of the parent cells. By age is meant the time between the start of growth of the parent culture and the transfer of the inoculum in the subculture. When amino acids are the source of nitrogen the result is as shown in Fig. 15. The absence of lag with a young culture and the increase in lag with age has of course long been known qualitatively, and is confirmed quantitatively by this figure.

When ammonium sulphate, on the other hand, is the nitrogen source, the lag-age relation is as illustrated in Figs. 16*a*, 16*b*, 16*c*, and 16*d*. The explanation of the minimum, observable in the curves, is as follows. In the ammonium sulphate medium a diffusible intermediate escapes into the solution. In the ordinary procedure of subculture the cells transferred to the new medium carry with them a certain volume of the original solution. When the inoculum consists

† R. M. Lodge and C. N. Hinshelwood, *J. Chem. Soc.*, 1943, 213.

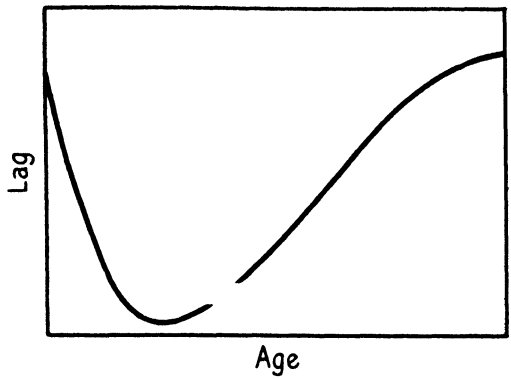


FIG. 16*a*. General form of lag-age relation in ammonium sulphate medium.

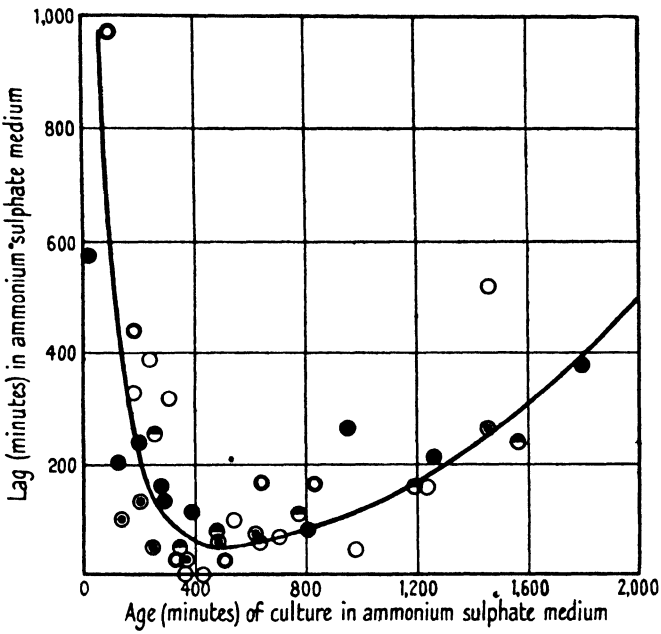


FIG. 16*b*. Experimental lag-age curves for various parent cultures.

of very young cells, there is not enough of the diffusible intermediate transferred to satisfy needs in the new medium, and time is required for it to be produced. With older inocula, enough is transferred to supply these needs, and the lag falls to a minimum. The subsequent

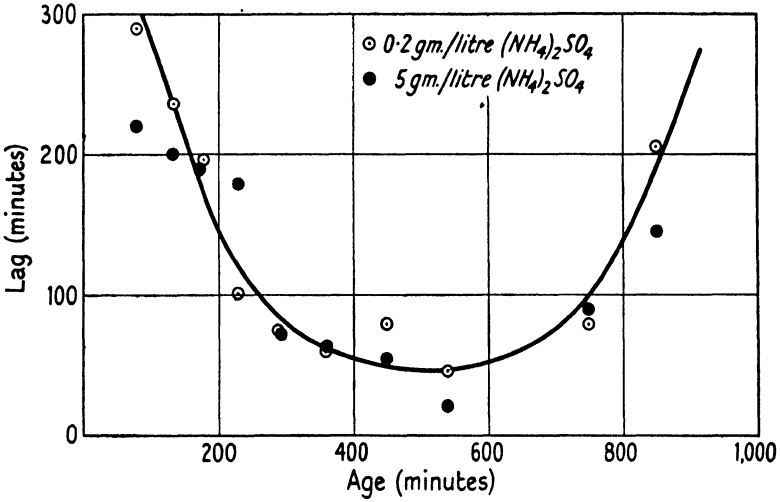


FIG. 16*c*. Influence of ammonium sulphate concentration on lag-age relation. (A. M. James.)

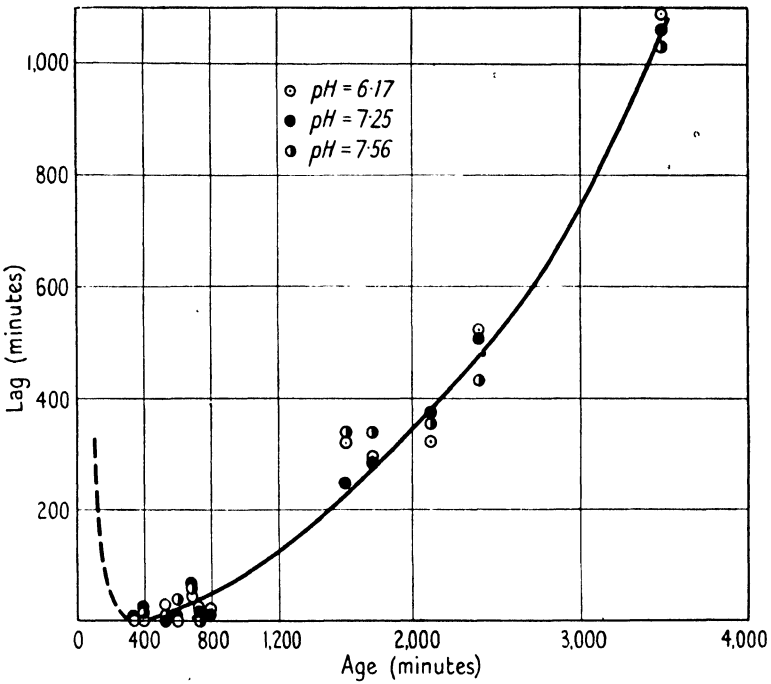


FIG. 16*d*. Influence of pH on lag-age relation. (A. R. Peacocke.)

rise in the value of the lag on further ageing is of course analogous to what occurs in the amino acid medium. The explanation of the *early lag* (i.e. that corresponding to the falling part of the curve in Fig. 16) is confirmed by the fact that small quantities of solution separated (by filtration or centrifuging) from a fully-grown culture and added to young cultures completely remove the delay in growth.

There is an obvious relationship between this influence of filtrate on the lag phase and observations which have been made in other connexions upon diffusible co-enzymes. M. Sahyun, P. Beard, E. W. Schultz, J. Snow, and E. Cross† describe a 'growth activator' for *Bact. coli*. Diffusible co-enzymes have been postulated in connexion with the deaminases of *Bact. coli* by E. F. Gale and M. Stephenson‡ and in connexion with the lactic acid dehydrogenase of the same organism by J. Yudkin.§ With the deaminases of *Clostridium Welchii* there is a variation of activity with dilution which also strongly suggests a diffusible substance capable of escaping from the cells in the earlier stages of growth.‖

The early lag phenomenon is clearly interpretable in terms of a diffusible intermediate involved in the utilization of the simpler nitrogen sources. The increased lag which accompanies ageing beyond the point of minimal lag may be explained by all or any of the following factors: (*a*) chemical decay of active intermediates in the cells or the surrounding medium; (*b*) loss by diffusion from the cell of intermediates; (*c*) inactivation of cell enzymes by processes such as denaturing of proteins; (*d*) chemical saturation of free radical-ends of macromolecules so that they become incapable of further expansion. The relative importance of these is uncertain: (*b*) undoubtedly does occur and (*a*) may very well do; (*c*) and (*d*) stand possibly in a fairly close relation. The activities of individual enzymes, as shown by tests on washed cells, undoubtedly decline as ageing progresses, as was mentioned on p. 34, and the time scale of this process is in fact comparable with that of the lag development. An example is given in Fig. 17, which shows the variation of the catalase activity of *Bact. lactis aerogenes* (measurements of E. H. Cole) and may be compared with Fig. 15. One observation which suggests, though it does not by any means prove, the dispersal of supplies of intermediate during the first stages of the development

† *J. Inf. Dis.*, 1936, **58**, 28. ‡ *Biochem. J.*, 1938, **32**, 392.
§ Ibid., 1937, **31**, 865. ‖ D. D. Woods and A. R. Trim, ibid., 1942, **36**, 501.

of late lag is that the value of the latter often rises fairly rapidly at first and then settles down to a more or less steady value. This does not suggest a progressive decay of cell substance so much as a fairly rapid loss of intermediates. On the other hand, at still greater ages the lag eventually increases rapidly and the cells die, so that the

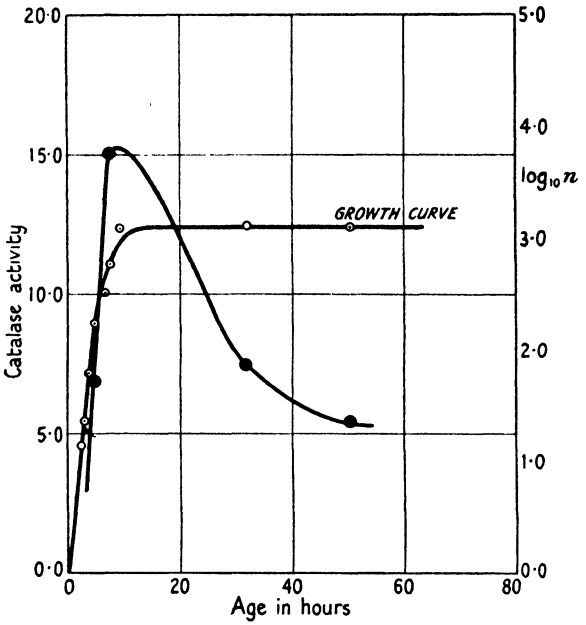


FIG. 17. Variation of catalase activity of *Bact. lactis aerogenes* with age of cells.

inactivation of actual cell material does in the end occur. The rate of 'ageing' of the cells as represented by the increase of lag is a function of the medium and seems to be increased by acidity. For example *Bact. lactis aerogenes* develops 'late lag' less rapidly in a glycerol medium† than in a glucose medium. In the former little acid is formed, in the latter considerable amounts. Although it is of biological interest rather than chemical, it might be mentioned that the lag over a space of many days may actually show slight rises and falls in some cases. This is probably connected with the fact that some of the cells die and suffer destruction, and that degradation products from their substance become available to permit a small

† A. H. Fogg and R. M. Lodge (unpublished).

renewal of growth in some of the others.† The effect is not important, but it may be mentioned as an example of the difficulties which can beset experimental work.

The importance of substances carried over from an old medium to a new one (mentioned above for the example of *Bact. lactis aerogenes*) raises the question whether perhaps all bacteria which use very simple compounds may not be dependent upon small stores of more complex substances handed on with successive inocula from one growth cycle to the next. This idea can be to some extent negatived by the observation that the cells may in certain cases be washed free of filtrate from the previous culture cycle and are still able to grow in the new medium, even if with an increased lag—during which they themselves build up the necessary concentration of the intermediates. It might be argued that some of the supply of intermediate cannot be washed out of the cell. But this would mean that the intermediate in question could be more appropriately regarded as part of the cell substance than as a separable compound, and no one would deny that in respect of its major material basis the cell is entirely dependent upon the reproduction of existing patterns.

4. Lag in relation to the number of cells transferred in the inoculum

Numerous qualitative references to the influence of inoculum size on lag occur in the earlier literature.‡ The phenomenon is best understood in relation to the early lag phenomenon discussed in the last section. If the termination of the lag demands a certain minimum concentration of a diffusible intermediate, and if insufficient of this is transferred with the actual medium accompanying the inoculum, then the cells must build it up in the new environment for themselves. Since they all form the intermediate and pour it out into the medium to make a common store, the more of them there are to contribute their quota the sooner is the critical concentration built up.

Bact. lactis aerogenes shows a very marked dependence of lag upon number of cells in the inoculum when growth takes place in the ammonium sulphate medium referred to above. When amino acids are used instead of ammonium sulphate the early lag phenomenon

† For an example of this 'cannibalism' of cells, see E. A. Steinhaus and J. M. Birkeland, *J. Bact.*, 1939, **38**, 249, who find that the 'senescent phase' of *Serratia marcescans* may continue for months, with rises and falls of population.

‡ Topley and Wilson, *Bacteriology*.

is absent, a fact which shows growth to be much less dependent upon diffusible intermediates in the solution: and accordingly there is little or no influence of inoculum size on the lag. The contrast is illustrated by the following numbers.

TABLE I

Growth in ammonium sulphate medium

Inoculum size, millions per c.c.	*Lag (min.) observed*	*Lag (min.) calculated— see next section*
3·18	136	136
0·42	804	640
0·18	1000	950
0·069	1220	1220

Growth in asparagine medium

Inoculum size, millions per c.c.	*Lag (min.)*
37·0	350
18·5	490
7·4	350
2·2	338
0·75	345

5. Quantitative theory of early lag and influence of inoculum size†

It will be assumed that the lag ends when the concentration, c, of some active substance reaches in each cell a critical value c'. We write

$$c = \alpha v + \beta n_0 t + \gamma t,$$

where v is the volume of the old medium transferred with the inoculum, and αv is the concentration of the active substance thereby set up. α will be a function of the count n_1 of the parent medium (so long as the bacteria have not been separated from their medium by centrifuging or otherwise), and n_0 is the number of cells per unit volume of the new medium, so that $\beta n_0 t$ is the contribution to c which they will have made in time t by generating active substance in the medium. The cells will retain some of the active substance which they individually build up, so that a term γt is added to represent

† R. M. Lodge and C. N. Hinshelwood. *J. Chem. Soc.*, 1943, 213.

what is built up in a given cell without the contribution of the others. The simple summation of the last two terms can only be regarded as an approximation, but it will probably not be far from the truth.

When $c = c'$, $t = L$, where L is the lag, whence

$$L = (c'/\beta - \alpha v/\beta)/(n_0 + \gamma/\beta).$$

From this equation may be deduced the following:

(*a*) When n_0 is constant but v varies, as when filtrate from grown cultures is added with the inoculum, the lag should decrease linearly with increase of v.

(*b*) When v is constant and n_0 varies, L assumes the form

$$L = \text{const.}/(n_0 + \text{const.}),$$

which is in fact used to express the numbers in the last column of Table I (previous section). (The independent variation of n_0 is effected by centrifuging cells from their medium and then employing cells and medium in any required amounts.)

(*c*) In the normal subculture experiment the variation both of v and n_0 must be considered.

The requirement (*a*) is, at any rate qualitatively, satisfied, as illustrated by the following numbers:

n_0 constant:					
Added filtrate (v)	0	0·1	0·25	0·5	1·0
Lag	182	106	46	70	12

The form (*b*) is shown to be approximately correct by the Table referred to.

Some measurements illustrating (*c*) are shown in Fig. 18. Two separate parent cultures, of different total count, indicated on the diagram by n_1 (in arbitrary units) were used as inocula for the two series plotted. n_0 is the actual inoculum size (arbitrary units), v varying in proportion to it, since no adjustment by use of washed cells or of filtrate was made. From a set of measurements similar to those given in Table I of the last section (constant v) the value of γ/β is calculated. The upper curve in Fig. 18 is then expressible by the equation

$$L = (448 - 2350v)/(n_0 + 0{\cdot}258).$$

For the lower curve, to obtain the correct value of the lag at the smallest value of n_0, α/β must be taken as 2950. With these various

constants the experimental results can be expressed as well as can be expected having regard to the inherent experimental difficulties.

$n_1 = 132$

v	n_0	*lag (obs.)*	*lag (calc.)*
0·1	0·57	185	185
0·2	1·13	0	0
0·3	1·70	0	0
0·5	2·83	0	0

$n_1 = 960$

v	n_0	*lag (obs.)*	*lag (calc.)*
0·01	0·40	450	645
0·03	1·20	260	260
0·10	4·0	50	50
0·20	8·0	10	0

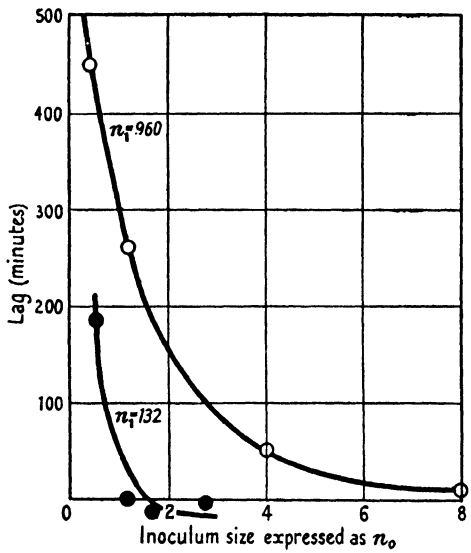


FIG. 18. Dependence of lag upon number of cells in inoculum.

Since β should be a constant, the values of α/β will vary as α. To the above values is added one for a very young parent culture and the set is tabulated below:

n_1	25	132	960
α/β	660	2950	2350

This indicates that the amount of active substance in the medium of the parent culture rises at first and then slowly declines as might be expected.

The general outlines of the above theory seem to be in good accord

with experiment. The assumption that the lag ends when a definite critical concentration of an active intermediate is built up needs, however, further examination before it can be accepted in its literal form. It may, on the one hand, simply amount to an approximation which is nearly enough satisfied in practice: or, on the other hand, it may be correct in principle. If the latter can be said, then we need an interpretation of the kinetic mechanism by which such behaviour could be conditioned. Critical concentration limits below which reactions do not occur and above which they occur with explosively great speed are indeed known in chemical kinetics, but are found in connexion with systems which do not at first sight appear to resemble anything likely to exist in the cell. The matter will be left open here, where we shall not assume more than that a convenient approximation has been used.

6. Theory of late lag

In the earlier literature† various views about the nature of the lag phase are expressed. Penfold's is the most general and the most satisfactory in that it places the primary emphasis on the step-like character of cell processes, and upon the necessity for the building up of the right intermediates. It is sometimes quoted in contrast to the so-called 'Bios' hypothesis, which postulates the need for an adequate concentration of an essential growth factor in the medium. Chesney's hypothesis regards the lag as the time required for recovery from the toxic effects of the previous environment. In the light of the foregoing discussion it would seem that all these hypotheses contain elements of truth. The facts described in §§ 3, 4, and 5 of this chapter leave no doubt that in certain circumstances the lag of a culture may be determined by the time required to build up an adequate concentration of a diffusible substance in the medium. On the other hand, they show that even for one single organism the importance of this factor may vary widely according to the precise nature of the medium in which growth occurs. No hypothesis resting wholly upon the idea of diffusible growth substances can claim any general validity, though it may perfectly correctly describe a kind of behaviour which is in fact encountered. Moreover, it correlates interesting facts about the influence of inoculum size, for those examples where such facts are met. But they are not met universally.

† See Topley and Wilson, loc. cit.; also, Winslow and Walker, loc. cit.

It would be of interest to extend studies such as those described in the sections referred to so as to include a whole range of organisms and media.

There is perhaps no real incompatibility between what has been described as Penfold's hypothesis and that of Chesney. The former lays more stress upon the disturbance of the balance of the intermediate substances, the latter upon the deleterious action of toxins. The whole approach to the subject which has been developed in the preceding chapters is consistent with the general idea expressed by Penfold, but it leaves plenty of room also for the factors upon which Chesney lays stress. As has been said, during the stationary phase the steady concentrations of the intermediates established in the logarithmic phase will be disturbed, and, at the same time there will be a gradual decay of the enzyme activities, possibly attended with an actual denaturation or destruction of the enzyme substance. The question is not so much which hypothesis is correct, as what is the relative importance of the two factors in each given instance. This is a matter for detailed experiment with various organisms, which are allowed to age under different conditions in different media. One might well imagine that in a medium such as a carefully chosen buffer solution loss of intermediates, at least for a time, would constitute the more serious cause of ageing, while in a very acid medium actual enzyme decay would soon become the dominant factor. Experiments on the rate of development of lag, and rate of decay of enzyme activities, carried out systematically under a wide range of conditions are much to be desired.†

Theories which involve terms such as 'inertia', or 'rejuvenesc[illegible] may possibly have some descriptive value when handled in their

† *Note added during publication.* Since this chapter was written some experiments have been made by E. H. Cole and A. M. James on the development of lag in ageing cultures of *Bact. lactis aerogenes*, with simultaneous measurements on the decay of catalase and dehydrogenase activity. The cultures were allowed to age both in the growth medium (glucose, ammonium sulphate, phosphate) and in saline buffered to various known pH values. It was confirmed that the principal factor causing the rather rapid decay of the enzymes in the former was the highly acid pH which had been developed. Another provisional conclusion is that the lag develops more rapidly in the early stages of ageing than would be accounted for by the decay of either of the two enzymes studied. This may be of no particular significance as far as catalase is concerned, but, as will appear later, there is a close correlation between growth and dehydrogenase activity. It would seem, therefore, that the increase of lag, initially at least, is due rather to the dispersal of the intermediates and the destruction of the steady state in the reaction sequence than to material damage to the fabric of the cell. (See also pp. 81 and 83.)

proper context by their own authors, but they do not come within the scope of the present considerations.

One further hypothesis must, however, be considered. It is that which has been referred to as the selection hypothesis. According to this, there is a wide range of variation in the power of individual cells to grow. During the lag, although the total number of organisms may show only a negligibly or immeasurably small increase, there is a great multiplication of certain more rapidly growing variants initially present in very minute proportion. What must be conceded at once is that if the initial strain is mixed, then such a selection of the more rapidly multiplying types will in fact occur. But it is by no means permissible to assume without evidence that in all cultures during the lag phase the bulk of the population constitute mere passive spectators of events in which only a favoured few participate. The extent to which such a phenomenon occurs in any given kind of example is, however, determinable by experiment with the help of appropriate methods, one of which will be described.

When a culture is allowed to age considerably, many of the cells die. Quite evidently, then, if an inoculum is taken from such a mixture of living and dead cells an extreme form of selection must take place. Suppose the total number of cells, living and non-living, is initially n_0', of which n_0 are living, and suppose n_0 is small compared with n_0'. Then if the lag is determined by extrapolation to n_0' of the logarithmic part of a growth curve, the time taken for the living cells to increase in number from n_0 to n_0' will be reckoned as part of the lag phase. The value so found may be termed the *apparent lag* in contrast with the *true lag* which is the time between inoculation and the onset of growth among the living cells themselves. In an analogous way, even if the bulk of the population consists not of dead cells but merely of slow-growing ones, the apparent lag of the whole culture will be quite different from the true lags of the individuals which contribute most to the observed growth. Now it can be shown that in certain cultures exhibiting lag, the true lag and the apparent lag are nearly equal. This means that the apparent lag, in these examples, is not in fact the time required for a small minority of the population to grow up to the threshold of measurability: most of the organisms are therefore alive and endowed with at least approximately equal powers of multiplication (although a certain statistical variation about the mean is not excluded). In other

examples it appears equally clearly that the apparent lag is due to the presence of a majority of dead cells in the inoculum. It is, therefore, evident that the intermediate case of cultures containing many cells of low reproductive power is in principle very probable. But it is quite incorrect to suppose that this constitutes in any sense a general explanation of lag phenomena.

It will be convenient to consider the means of distinguishing true and apparent lag in a separate section.

7. True and apparent lag†

Let n_0 be the initial number of living cells, x_0 the number of dead cells, n the total number at time t, and L the true lag of the living cells. The death of organisms during the period of observation will be neglected, so that we have

$$n = x_0 + n_0 e^{k(t-L)},$$

whence

$$\frac{d\ln n}{dt} = \frac{1}{n}\frac{dn}{dt} = \frac{kn_0 e^{k(t-L)}}{n} = \frac{k(n-x_0)}{n}.$$

If all the cells had been living and had grown at the same rate, $d\ln n/dt$ would have been equal to k: so that we shall in the actual case call it k_{apparent}. From the above equation then

$$x_0/n = (1-k_{\text{app}}/k).$$

The true and apparent mean generation times are in the inverse ratios of the corresponding values of k.

In the experimental determination of a growth curve it is usual to employ a small inoculum, in which case the values of n in the range of the actual measurements are very much greater than either n_0 or x_0. When this applies the last equation shows that $(1-k_{\text{app}}/k)$ approaches zero; in other words that the true and apparent values of the growth rate are indistinguishable. If, however, the normal method of procedure is modified so that a very large inoculum is employed, and if x_0/n_0 is appreciable, then k_{app} will differ significantly from k, and, moreover, the value will vary with n, so that the logarithmic law of growth will not be even approximately followed. The form of the deviation allows the calculation of x_0/n_0. The following is an example of the application of the method.

A culture of *Bact. lactis aerogenes* showing considerable lag was inoculated into broth. In one experiment a very small inoculum was

† R. M. Lodge and C. N. Hinshelwood, *J. Chem. Soc.*, 1943, 213.

employed and the true value of the mean generation time was measured when the logarithmic phase was established: the value was 22·2 minutes. In a second experiment a heavy inoculum, about a hundred times greater than before, was used so that n_0+x_0 remained comparable with n over most of the range of observation. The growth was, however, still logarithmic, with a mean generation time of 22·7 minutes, measured at the point where $n = 90$. From the last equation, therefore, $x_0/n = 1-\frac{22{\cdot}2}{22{\cdot}7}$, whence it follows that x_0 does not exceed about 2 in the arbitrary units employed. n_0 in those same units being 48, it appears that about 96 per cent. of the original inoculum was living.

The same method applied in a slightly different way is exemplified by the following. A culture of the same bacterium with a lag of 300 minutes was transferred to an asparagine-glucose medium in a series of experiments with different inoculum sizes. Growth curves were determined and, from their slope at a given value of n, the apparent mean generation times were measured. These were compared with the values calculated from the equation for k_{app} (*a*) on the assumption that all the cells were alive and that the true lag and the apparent lag were identical, and (*b*) on the assumption that there was no true lag and that the whole of the observed lag was due to the presence of non-growing cells. The selection of typical numbers quoted below shows that for the culture in question (*a*) comes very much nearer to the truth than (*b*).

	Mean generation times at value of $n = 220$		
Inoculum size	*Calc.* (*a*)	*Calc.* (*b*)	*Observed*
6·5	29·3	30	28
18	29·3	32	29
54	29·3	39	31·5
171	29·3	130	33

Under unfavourable conditions cells do in fact die and as a result the apparent lag increases with time. It sometimes happens that the whole increase can be attributed to this cause, as the following consideration shows. If we *assume* that under rather markedly adverse conditions the increasing apparent lag is due to the drop in n_0, then we can follow the course of n_0 as ageing of the culture proceeds.

It is then easy to predict the time at which n_0 will have fallen to less than one cell per culture tube. At this point, of course, the statistical laws of growth or death no longer apply: nevertheless, we can see that some of the culture tubes, if not most, are likely to contain no living cell at all, and that further ageing should result, not in further prolongation of the apparent lag, but in complete sterility. This is in fact precisely what can sometimes be observed.

All varieties of behaviour thus seem to be represented. But it is as well to restrict the term lag to what we have termed true lag, and to regard non-viability not as a cause of lag, but as a source of error in the measurement of the true lag.

8. The stationary phase ; maximum bacterial population

Before discussing the logarithmic growth phase, it will for various reasons be convenient to discuss those factors which lead to the ultimate termination of growth, and which determine the maximum population which the medium can support.

Various suggestions are made in the older literature about the reasons for the cessation of growth at the end of the logarithmic phase.† One obvious possibility is exhaustion of necessary nutrient materials in the medium. Some investigators have, however, observed that the organisms can be separated from the fully-grown culture and the filtrate used, after various treatments such as boiling, to support further growth on reinoculation. Thus exhaustion cannot invariably be the controlling factor. According to Bail, the cells have filled all the available 'biological space', and other authors assume that the cells have consumed all the available oxygen, or that accumulated toxic products, or unfavourable pH, are responsible for the inhibition of further growth.

The matter becomes clearer in the light of quantitative experiments on the dependence of the maximum population, which will be designated n_s, on various factors, such as the foodstuff concentration and the pH. These observations show that according to circumstances either exhaustion of food supplies, on the one hand, or the accumulation of toxic products, on the other, may become the limiting factor, even with one and the same organism in the same type of medium.

† Topley and Wilson, op. cit., for references to the work of Penfold, Graham-Smith, Kojima, and others.

It will be convenient first to consider what effect dilution of the medium should have upon n_s (1) when cessation of growth is governed by exhaustion, and (2) when it is governed by the accumulation of products antagonistic to further multiplication.

First suppose growth continues until the concentration, g, of a given foodstuff falls to zero. As we have already seen, the growth rate does not drop until the exhaustion is almost complete. Hence the equation $dn/dt = kn$ describes the growth rate over practically the whole course. If the average rate of consumption is f per organism, then $-dg/dt = fn$, and

$$g = g_0 - f\int_0^t n\,dt.$$

When $g = 0$, $n = n_s$, and neglecting n_0, the initial count, compared with n_s, we have $n = g_0 k/f$. In other words the maximum population should be directly proportional to the initial concentration of the foodstuff.

If, on the other hand, toxic products play an important part, their influence on the rate of growth must be expressed. It will be shown later that the reduction in growth rate is often linearly proportional to the concentration of the inhibitor. Thus

$$dn/dt = kn(1 - ac),$$

where c is the inhibitor concentration and a is a constant. If, on the average, the toxic material is formed at a rate r per organism, then $dc/dt = nr$. Therefore

$$c = r\int_0^t n\,dt$$

and

$$dn/dt = kn\left(1 - ar\int_0^t n\,dt\right).$$

$\int n\,dt$ is the area under the curve of number against time. Dilution of the medium *per se* does not seriously affect the growth rate, and at the end of a given time the concentration of toxic products formed will at least not be greater than in a more concentrated medium. Thus on dilution the value of n_s will not be decreased.

In the light of these considerations we may examine some observations on *Bact. lactis aerogenes*,† which has formed the subject of a fairly detailed study.

† S. Dagley and C. N. Hinshelwood, *J. Chem. Soc.*, 1938, 1930; R. M. Lodge and C. N. Hinshelwood, ibid., 1939, 1683; 1943, 208.

In the lactose-ammonium tartrate medium of Winslow, Walker, and Sutermeister† the variation of n_s with concentration of lactose and of tartrate respectively is shown in Fig. 19. The relation is evidently nearly linear over most of the range, a result showing that exhaustion is the principal factor. But the slight curvature of the line representing the results with the tartrate, and the pronounced bend corresponding to the higher lactose concentrations show that

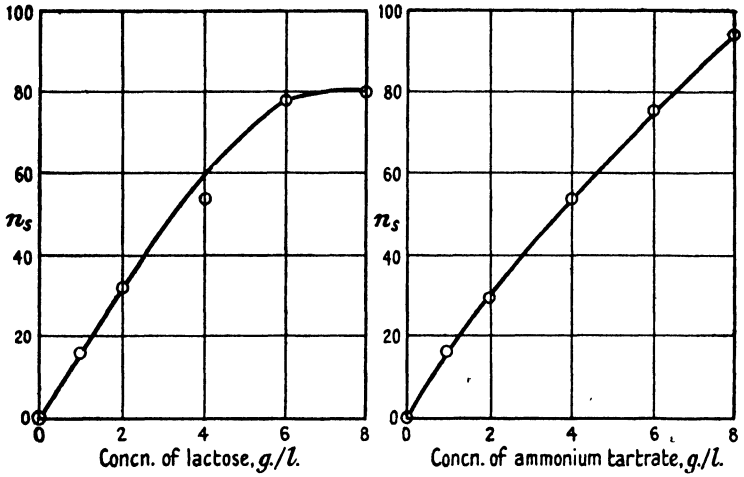


FIG. 19. Influence of concentration on total population.

toxic products are exerting an influence which begins to be important at the higher concentrations. Similarly in a medium containing glucose and ammonium sulphate n_s varies linearly with the glucose-concentration over a fairly wide range, and finally tends to become independent of it.

That the development of acidity in the medium (for example by fermentation of carbohydrate) is in itself capable of causing a cessation of growth is shown by the influence of initial pH on the value of n_s. In Fig. 20 it is evident that the value of n_s drops rapidly towards zero if the initial pH is outside a certain range.

The comparison of the two curves in the figure is itself of some interest. The higher curve, which has no horizontal plateau, refers to a medium with a high glucose concentration. The stationary population is seen to vary with the pH over the whole range, indicating

† *J. Bact.*, 1932, **24**, 185.

that pH is always a limiting factor. The lower curve refers to a dilute glucose solution. It possesses a horizontal plateau, showing that, over a certain range, the pH ceases to be the limiting factor, and that cessation of growth is determined in this region by the exhaustion of glucose. The converse case is illustrated by the following results.

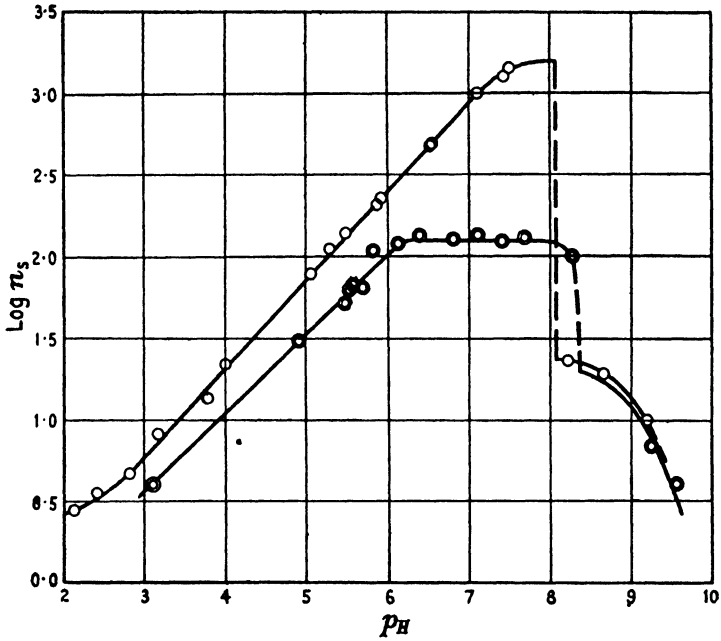


FIG. 20. Influence of pH on total population in two glucose-phosphate media.

In a phosphate-glucose medium of favourable pH the value of n_s is strongly dependent on the glucose concentration. In a similar medium of unfavourable pH exhaustion of glucose ceases to be the limiting factor and the value of n_s becomes independent of glucose concentration.

Relative values of n_s

	Glucose 24·6 *g./l.*	*Glucose* 0·99 *g./l.*
pH 7·11	1000	172
pH 5·20	95	95

When *Bact. lactis aerogenes* is grown in glucose-amino acid media, n_s in general is much increased by aeration of the medium, but to an

extent which varies with the particular nitrogen source employed. The effect of the aeration seems to be to aid the removal of an inhibitor, formed during growth, which, if it remains in the medium, can become the limiting factor and bring the logarithmic phase to an end.

A large volume of admirable results on the total populations of *Bact. coli* and of *Bacillus subtilis*, in different media, is given by J. Monod.† He finds an accurately linear relation between the concentration of the foodstuff and the value of n_s. The following are typical: for *Bacillus subtilis* growing on a medium containing saccharose he finds:

Concentration, c. mg./l.	*Relative* n_s	n_s/c
300	82·5	27·5
250	68·0	27·2
200	56·5	28·3
150	38·3	25·5
100	26·2	26·2
50	15·5	31·0
25	8·0	32·0

There remains no doubt that under the conditions of his experiments exhaustion was always the limiting factor.

But it must be observed that the maximum concentrations used by Monod are small in comparison with those in which bacteria will still grow readily. For example, the concentration of lactose corresponding to the sharp bend in the curve in Fig. 19 is of the order of tenfold the highest employed by Monod. While, therefore, his results constitute the best evidence available of the exactness with which concentration in appropriate circumstances determines n_s, they do not give quite the whole picture, which remains: that exhaustion on the one hand, or adverse changes in the medium on the other, each according to circumstances, is capable of becoming the limiting factor. The general relation is summarized in Fig. 21.

9. The logarithmic phase of growth

In the logarithmic phase the autosynthetic activity of the cell material achieves its full expression.

With a bacterial culture, growth may be represented either in terms of total bacterial *mass*, m, or, alternatively, in terms of the

† J. Monod, *La Croissance des Cultures Bactériennes*, Paris, 1942.

number of cells, n. Under ideal conditions, when division occurs at a perfectly standard cell size, these two methods are identical. It is true that, within a given cell, there must be a small alternation of conditions as it increases from the size of a newly-formed cell to that of one just about to divide, but this effect, even though it is itself responsible for the occurrence of division, is, from the point of view

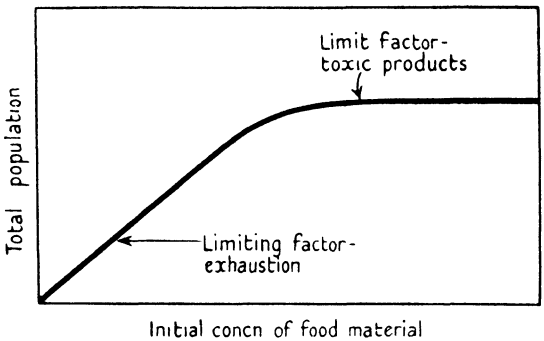


FIG. 21. Total population and concentration. General form of relation.

of growth rate, averaged out over the whole assemblage of cells making up the culture. Thus, with a constant environment, the two laws,

$$n = n_0 e^{kt} \quad \text{and} \quad m = m_0 e^{kt},$$

express the same thing.

In practice the logarithmic law is found to be a very good approximation over quite a wide range of conditions, both when growth is measured by microscopic counts of cell numbers, and when it is determined by methods such as turbidimetric observations which depend essentially upon total mass.†

The reasons why, on the one hand, the logarithmic law lacks an absolute character, and, on the other hand, why it is such a good approximation are interesting. It lacks absolute character for the following reasons. First, the concentration of foodstuff in the medium must change as the cells grow. Secondly, products formed by the cells pass into the medium and exert effects, sometimes inhibitory effects, as they accumulate. Thirdly, not every cell formed by the division of a parent survives to divide itself: thus although the logarithmic law might be followed by the number of living cells,

† J. Monod, op. cit.

it may not be followed by the total number. Fourthly, the conditions for cell division vary somewhat according to the state of the medium and, therefore, according to the stage of the growth: thus the actual size of the cells varies during the period of active multiplication, and the logarithmic law may describe either the total number or the total mass, but not both simultaneously. Fifthly, the logarithmic form can only be expected to apply in a truly steady state, when all the cell enzymes have settled down to constant proportions: that is to say, when the cells are fully adapted to the medium in which they are growing. When transferred to new media, cultures tend to exhibit growth curves of non-logarithmic and sometimes erratic form.

Over quite wide ranges of experimentally realizable conditions, however, these factors are unimportant, so that the law usually works well enough in practice. First, as will appear in the next section, growth rate is usually very nearly independent of foodstuff concentration over wide limits, so that the medium can become almost completely exhausted before a slowing down of growth sets in. Secondly, there is usually a range of concentration within which toxic substances exert little effect, but outside which their action increases steeply.

Thirdly, the mortality under favourable conditions may be very low. According to Wilson† with some organisms, even during the logarithmic phase, the percentage of new cells which survive seldom exceeds 90 per cent. of the total. Kelly and Rahn,‡ however, made direct observations on the division of individual cells of various bacterial species and found that under favourable conditions all cells continued to divide. Even with Wilson's figure of 90 per cent. the departure from the logarithmic law for total numbers would not be serious.

Fourthly, although quite important variations in cell size occur (and are discussed at greater length in Chapter X), under the more usual conditions they are confined to the extreme ends of the growth cycle, and do not play an outstanding part during the greater part of the logarithmic phase.

The various factors which cause departures from the logarithmic law usually rise from negligibility to importance in the rather short period when the bacterial count is becoming greater than a certain

† G. S. Wilson, *J. Bact.*, 1922, **7**, 405.
‡ G. D. Kelly and O. Rahn, *J. Bact.*, 1932, **23**, 147.

limit. In this region the foodstuff concentration falls, the toxic products accumulate, and division conditions change. The result is a rather abrupt slowing down of growth as shown in Fig. 9. The normal method of plotting emphasizes this abruptness: the ordinates represent the logarithm of the number of cells (or of the bacterial mass), and equal increments of the ordinate therefore represent rapidly increasing increments of cell substance produced, which in turn mean rapidly increasing consumption of foodstuff or production of toxic products. If a growth curve is observed over ten mean generation times, the amount of nutrient consumed during the last of them is approximately equal to that consumed over the whole of the other nine.

Attempts have sometimes been made to represent the whole of the growth curve, including the transition to the stationary phase, by a single equation. Some of these are discussed by Monod.† In general the problem reduces to replacing k in the logarithmic equation by a function of m or n, and determining this function. Thus instead of

$$\frac{dm}{dt} = km \tag{1}$$

we write

$$\frac{dm}{dt} = f(m)m, \tag{2}$$

or regarding k itself as a variable we write $k = f(m)$.

Verhulst proposed a law of growth of the form

$$\frac{dm}{dt} = Am\frac{B-m}{B}.$$

When m is small in comparison with B this reduces to $dm/dt = Am$ and if $A = k$ this is the original simple law: when m grows to a value B the rate of increase becomes zero so that a limiting population is reached.

Equations analogous to those of autocatalytic chemical reactions attended by consumption of a finite amount of substance have been applied to growth phenomena in general, for example,

$$\frac{dm}{dt} = Km(a-m).$$

It seems quite clear, however, that neither of these equations can have more than a qualitative significance.

† Op. cit.

From what has been said about the reasons for the onset of the stationary phase, it follows that no single equation can be expected to give a correct analytical expression of the transition. Sometimes growth stops because foodstuff is exhausted, sometimes because toxic products have accumulated. On occasion, these two effects will be superposed. For convenience, however, the two cases will be considered separately.

When exhaustion is the controlling factor we must first know k as a function of c, the concentration of the foodstuff, and then in turn the concentration c itself must be expressed in terms of m. This case has been considered by Teissier and by Monod. The reasonable assumption is made that the rate of fall in c is always proportional to the rate of increase in bacterial substance. Thus

$$-\frac{dc}{dt} = A\frac{dm}{dt}. \tag{3}$$

The variation of k with c is expressed in the form

$$k = k_0\frac{c}{c_1+c}, \tag{4}$$

which, as will appear in the next section, is well satisfied by experiment. c_1 is a constant.

Integration of (3) gives

$$c_0-c = A(m-m_0),$$

where c_0 is the initial concentration and m_0 the initial amount of bacterial substance. Substituting from the last in (4) and then introducing the value of k into (1) one obtains

$$\frac{dm}{dt} = k_0\left\{\frac{c_0+Am_0-Am}{c_1+c_0+Am_0-Am}\right\}m,$$

which gives on integration

$$k_0t = \frac{c_1+c_0+Am_0}{c_0+Am_0}\ln\frac{m}{m_0} - \frac{c_1}{c_0+Am_0}\ln\frac{c_0+Am_0-Am}{c_0}.$$

Under conditions where exhaustion is in fact the limiting factor this equation gives a correct expression of the course of growth. In practice c_1 is usually very small in comparison with c_0. Over a considerable range therefore it may be neglected: under which conditions the last equation reduces to

$$k_0t = \ln\frac{m}{m_0},$$

that is, to the simple logarithmic law. When, however, c drops to a value comparable with c_1 the law changes rapidly: at this point, however, exhaustion is nearly complete and the growth rate falls very quickly to zero.

The fall is, in experimentally measurable examples, so rapid as not to constitute a sensitive test of the precise form of the expression (4), which, however, is quite well founded theoretically, as will appear in the next section.

Equation (3) is a reasonable assumption and probably obeyed over quite wide ranges: but it is not necessarily true in general. Cells are capable of fermenting carbohydrates without actually growing. In principle, therefore, any foodstuff may be destroyed by the bacteria in a way unrelated to growth, and the proportion which suffers this fate may change as the composition of the medium changes. This factor is often enough unimportant, but it should be mentioned in so far as it helps to show the lack of fundamental significance in any one simple formula which purports to describe the whole course of the growth curve.

When accumulation of toxic products is the limiting factor, rather similar equations apply. We now have

$$\frac{dm}{dt} = km,$$

$$k = k_0 - f(x), \tag{5}$$

where x is the amount of toxic product.

During the period of active growth it is a reasonable approximation to write

$$\frac{dx}{dt} = B\frac{dm}{dt}, \tag{6}$$

but this is subject to reservations analogous to those made above in connexion with the linking of foodstuff consumption and growth. Accepting (6), however, we have

$$x = B(m - m_0),$$

the value of x being zero at the beginning of growth.

The form of the function in (5) can be studied by separate experiment on the addition of inhibitors. In general it is of rather complicated form, but often enough a linear relation, with $f(x) = B'x$, will serve: in which case the equations can be easily integrated. But, once again, it is evident that in principle no simple analytical form

really expresses the transition between the logarithmic and the stationary phase. Fortunately, however, the logarithmic phase is usually well enough defined over a long enough range to render possible the characterization of growth by a single value of k in the fundamental equation.

10. Growth rate and concentration of medium constituents

Over quite wide ranges the growth rate is almost independent of the concentration of the principal medium constituents. It is evident that the centres which deal with these substances are easily brought to a state of saturation. According to the discussion in I. 6 the relation between rate and concentration might be expected to follow an equation of form similar to an adsorption isotherm: in other words to be expressible by

$$\text{rate} = \frac{Ac}{1+bc},$$

where b is a constant, or by a more complex equation, describing, however, a curve of the same general shape.

If k_∞ is the limiting value of the rate constant when the concentration is large, the above may be written in the form

$$k = \frac{k_\infty c}{c_1+c},$$

c_1 being a new constant. c_1 has of course the dimensions of a concentration, and is in fact that concentration at which the value of k would lie half-way between zero and k_∞. The last equation is that which was introduced in the course of the discussion in the previous section. This was, therefore, simply a Langmuir isotherm rewritten.

The experimental determination of the relation between k, or mean generation time, and concentration is attended with not inconsiderable difficulty. The growth rate does not begin to diminish seriously until the medium is made very dilute indeed: and precisely at this point the stationary bacterial population becomes so small that observations have to be confined to a range too small for satisfactory accuracy. All observers seem to be agreed that the general course of the rate-concentration curve is given by an equation similar to that written above,† but none of the results really allow a choice

† W. J. Penfold and D. Norris, *J. Hyg.*, 1912, **12**, 527; S. Dagley and C. N. Hinshelwood, *J. Chem. Soc.*, 1938, 1930; J. Monod, op. cit.

between one of the Langmuir type, and one of rather more complicated form. What is, however, perfectly clear is the early saturation of the growth centres as the concentration rises.

The rate-concentration relation may be sought directly by determination of mean generation times at different initial concentrations (a method which encounters the difficulty mentioned above) or indirectly. An indirect method is as follows.† We first select a set of conditions such that growth is limited by exhaustion of foodstuff. A curve is then determined which gives the total bacterial population, n_s, as a function of the initial concentration of the particular medium constituent, the influence of which on the growth rate is to be measured. Now a growth curve is taken in a medium with an initial concentration c_0 of the constituent in question. A series of tangents to this curve are measured at various values of the count, n, in the region where the logarithmic phase is passing into the stationary phase. When the count is n, the amount of foodstuff consumed is that which would have been required to produce a total population of n, and this corresponds to a concentration c which can be read off from the first curve. The amount still unused at the point where the tangent is taken is therefore c_0-c. From the tangent the mean generation time, or the value of k, is obtained, and this value can then be plotted against c_0-c, and the required relation found. Fig. 22 shows a curve plotted by this method from experiments on the variation of phosphate concentration in cultures of *Bact. lactis aerogenes* in an artificial medium.

An extensive series of measurements on the variation of carbohydrate concentration is given by Monod partly for *Bacillus subtilis* but chiefly for *Bact. coli*. The following are some typical results.

Bact. coli *in synthetic medium*

Concentration of lactose, mg./litre	*Mean generation time, minutes*
2	860
9	550
29	85
43	65
90	53
114	56
137	57

The constant c_1 of the formula, representing the concentration at which the growth constant attains half its maximum value, gives a

† S. Dagley and C. N. Hinshelwood, *J. Chem. Soc.*, 1938, 1930.

convenient measure of the scale of the phenomenon. Some values given by Monod are as follows:

Bact. coli and glucose	4 mg./litre
Bact. coli and mannite	2 mg./litre
Bact. coli and lactose	20 mg./litre

The influence of different carbohydrates on the growth rate is rather diverse, but the results of measurements are of little significance unless they are made in relation with detailed studies of the

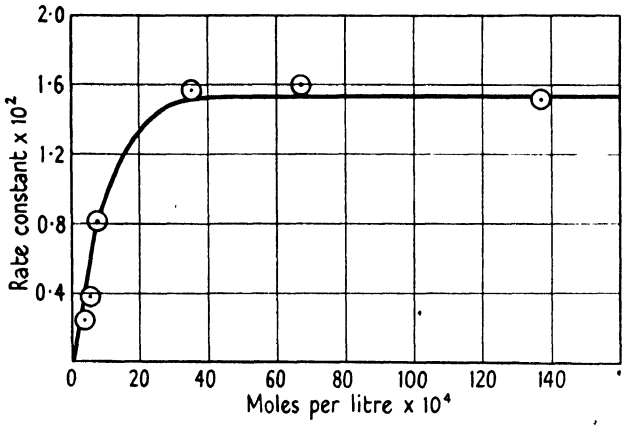


FIG. 22. Growth rate and substrate concentration.

state of adaptation of the bacteria to the source in question. Many coliform organisms, for example, grow considerably more slowly in glycerol or in lactose than in glucose when they are first transferred to these media. But after a period of adaptation they grow with equal facility in all three. The influence of the carbon source or of the nitrogen source on the growth rate will therefore be considered at a later stage and in connexion with the phenomenon of bacterial adaptation.

The influence of the concentration of a medium constituent of a somewhat different kind is shown in Fig. 23, which gives the growth rate of *Bact. lactis aerogenes* as a function of the carbon dioxide content of the air-stream aspirated continuously through the cultures in a synthetic medium.† It may be mentioned that when the amount

† Dagley and Hinshelwood, loc. cit.

of carbon dioxide in the air is 0·15 per cent. the concentration dissolved in pure water at the temperature of the experiments (40·0°) is 4×10^{-8} gram mol. per c.c.

It is interesting to note that the bacterial mechanism can function satisfactorily over a wide range of speeds. Various species will grow continuously and well at 40° in media in which the generation time varies from 18 minutes to over 200 minutes. Most of the information about the influence of the medium is of doubtful quantitative value,

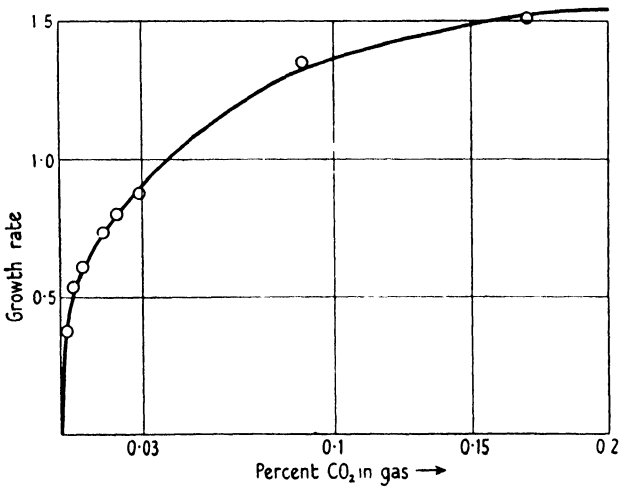


FIG. 23. Growth rate and carbon dioxide concentration.

since the growth rate, as explained above, depends upon the degree of adaptation. The limiting rate attained after thorough acclimatization to the new medium is not known in very many examples, but is certainly different for different media. The mean generation time of *Bact. lactis aerogenes* in bouillon is about 18 minutes. The minimum value to which it attains in a glucose-ammonium sulphate synthetic medium is about 32 minutes. If glucose is replaced by lactose, the generation time is at first very much longer, a fact which shows a new rate-determining step to have been added. After adaptation, however, the generation time falls to 32 minutes: one part of the mechanism has, therefore, improved in efficacy in such a way as no longer to constitute a bottleneck. But in each case there remains a step for which no further adaptive improvement is possible.

Often in the literature the effects of the medium on growth rate

and on stationary population are not clearly distinguished, all that is recorded being the abundance of growth after some chosen standard time. The variations in maximum population are often more important than the variations in rate. J. Gordon and J. W. M'Leod† classified amino acids as indifferent, favourable, or inhibitory. Considerable variations have been noted both in the rate of utilization of various amino acids and in the total populations: but even the latter depend upon the degree of adaptation. The aeration of bacterial cultures often has a marked influence on the total population, but not necessarily a corresponding one on the growth rate.‡ The following numbers illustrate the kind of effect observed.

Bact. lactis aerogenes *in glucose-amino acid-phosphate medium*

	Total population, millions per c.c.	
Amino acid	*Aerated*	*Unaerated*
Valine . .	1,530	69
Aspartic acid .	2,710	790
Asparagine .	6,000	1,250
Glutamic acid	1,950	62

In the early stages of growth, at least, the aeration made little difference to the generation time. The total population seems in such examples to be controlled in part by the formation of an inhibitor removable by cellular oxidation.

11. Influence of pH of medium on growth rate

The pH of the medium can hardly affect the total supply of any of the essential nutrient components, although it may cause wide changes in the availability of some of them. Its principal influence must be exerted upon the cell proteins. One might, therefore, have expected that changes of pH would have very marked effects on the growth rate and little on the final total population. Almost exactly the reverse of this is found, at least in one example, which there is no reason to suppose is not typical.

The profound influence of pH on n_s has already been considered (§ 8 and Fig. 20). In the experiments referred to, an adverse pH

† *J. Path. Bact.*, 1926, **29**, 13.

‡ M. Sahyun, P. Beard, E. W. Schultz, J. Snow, and E. Cross, *J. Inf. Dis.*, 1936, **58**, 28; R. M. Lodge and C. N. Hinshelwood, *J. Chem. Soc.*, 1943, 208; O. Rahn and G. L. Richardson, *J. Bact.*, 1942, **44**, 321.

reduced the total population almost to zero before any reduction occurred in the actual rate of the sparse residual growth. For example, at pH 6·45 a culture which ultimately grew to 190 million cells per c.c. reached 11·9 million in 1,000 minutes, while one in which the adverse initial pH of 8·3 reduced the total population to 12·5 million reached 11·9 million in about 900 minutes.

This rather surprising result should, of course, either be generalized, or set in a truer perspective, by more extensive experiments with other organisms in different media.

The influence of the pH upon n_s itself consists largely in changing the limiting factor from exhaustion of foodstuff to accumulation of inhibitors. In a medium of adverse pH the growth process becomes much more sensitive to the action of toxic agents formed during the logarithmic phase. But why this should be so, while the growth rate itself is not directly influenced, constitutes an interesting problem. One can, of course, assume that in the examples where this insensitiveness to pH of growth rate (and lag) is observed, there is a rate-determining process which happens not to share the general character of cell enzyme reactions in this respect: and that the other pH-sensitive cell reactions are merely not rate-determining for growth. If this is so, wider investigation of species and media should reveal more diverse kinds of behaviour. Certainly change in pH during the growth of *Bact. coli* causes a change in the enzyme content of the cells, as shown by the work of Gale and Epps† to which further reference will be made later.‡

† E. F. Gale and H. M. R. Epps, *Biochem. J.*, 1942, **36**, 600.

‡ p. 159.

IV

SOME GENERAL KINETIC ASPECTS OF CELL GROWTH

1. Introduction

In this chapter it is proposed to consider some simple propositions about the chemical kinetics of consecutive reactions, which may be applicable in the study of cell growth. These will later be discussed in connexion with actual problems of growth and adaptation, drug resistance of bacteria, and so on.

In the first instance we shall consider these matters in terms of the dynamics of individual cells. Having seen whether any useful progress can be made on this basis, we shall be in a better position to consider alternative working hypotheses such as those which refer all adaptive processes to the operation of natural selection.

The plan to be followed is to begin by an abstract discussion of a sequence of autosynthetic enzyme reactions: to consider how the proportions of the various enzymes are determined and how they may settle down to steady values: to investigate how the ratios may change with different conditions of reaction; and finally to compare the behaviour indicated with the phenomena of actual growth (lag phase, logarithmic phase) and adaptation.

In part the present chapter will constitute a source of reference for those which follow rather than a self-contained argument.

2. Elementary model of linked cell processes

(*a*) *Enzyme sequences.* We imagine a series of enzymes so linked that the product of the operation of each one constitutes the substrate for the next. Each one is assumed to undergo an autosynthetic reaction, with expansion of its own substance and simultaneous formation of a diffusible compound which can pass to the succeeding enzyme. The rate of growth of each is taken to be proportional to its own instantaneous amount and to be a function of the concentration of the substrate. This function will have the general form of an adsorption isotherm, giving direct proportionality to concentration for low concentrations and independence of rate and concentration for high ones. For the purposes of calculation it will be convenient to assume one or other of these limiting cases, rather than to introduce specific forms of function at this stage.

Let e_μ be the amount of the μth enzyme of the series. It will be expedient to define this, not as the amount in each cell, but as the total amount in the whole culture. e_μ, therefore, increases both when the number of cells, n, remains constant and the supply per cell increases, and also when the amount per cell is constant but the value of n becomes greater as a result of growth and division. de_μ/dt, therefore, represents the rate of expansion of bacterial substance, regardless of whether division is taking place or not.

For the expansion of the μth enzyme then, we write:

$$\frac{de_\mu}{dt} = k_\mu e_\mu c_{\mu-1} \tag{1}$$

or

$$\frac{de_\mu}{dt} = k_\mu e_\mu \tag{1 a}$$

according to the dependence on c_μ (the constants having of course a different meaning in the two cases). $c_{\mu-1}$ is the concentration of the substrate provided by the previous enzyme of the series.

The value of $c_{\mu-1}$ is determined by the balance of several factors: (1) the gross rate of production of the intermediate by the $(\mu-1)$th enzyme, (2) the rate of its consumption by the μth enzyme, and (3) the loss due to diffusion or to participation in irrelevant chemical reactions.

The calculations may be simplified, without effect on the essential results, by neglect of (2) in comparison with (3), i.e. the production of intermediates is supposed for simplicity to be rather prodigal, a fraction only being used in the direct reaction sequence.

For the net rate of production of the μth intermediate we write:

$$n\frac{dc_\mu}{dt} = k'_\mu e_\mu c_{\mu-1} - K_\mu c_\mu n \tag{2}$$

or the corresponding equation derived from (1 a). The terms in this equation require some comment. That on the left gives the rise in *concentration* multiplied by the number of cells: this term, therefore, represents the total *net* increase in amount of intermediate contained in all the cells. Strictly speaking, c_μ is thus expressed as the quantity per cell rather than per unit volume of substance, but, the average cell size being assumed constant, the appropriate numerical factor can be taken as incorporated in k'_μ and K_μ on the other side of the equation.

This net increase is balanced by the two terms on the right, the

first giving the gross production by all the bacterial substance and the second the total rate of loss. The latter will be proportional to the concentration prevailing, and also to the number of cells, since, if loss is by diffusion, then the rate is proportional to the area of wall, which in turn is proportional to n. Loss by irrelevant reactions will also be proportional to n.

The first term on the right is derived from equation (1), and expresses the condition that the enzyme yields its product in the process of self-synthesis. These two operations being proportional but not identical, the constant k_μ is replaced by k'_μ.

For the whole sequence of reactions, therefore, we shall have sets of equations of which the following are typical:

$$\frac{de_{\mu-1}}{dt} = k_{\mu-1} e_{\mu-1}, \tag{3}$$

$$n\frac{dc_{\mu-1}}{dt} = k'_{\mu-1} e_{\mu-1} - K_{\mu-1} c_{\mu-1} n, \tag{4}$$

$$\frac{de_\mu}{dt} = k_\mu e_\mu c_{\mu-1}. \tag{5}$$

These link the growth of the μth enzyme with that of the $(\mu-1)$th.

In (3) it is assumed that the enzyme is saturated with the substrate derived from preceding processes, whereas in (5) linear dependence of rate on substrate concentration is assumed. This assumption is not essential, of course, but it expresses the particular case which it will be useful to develop in this section.

When the steady state is established, that is during the logarithmic phase of growth, the concentrations of the various intermediates will assume stationary values in the sense discussed in I. 8. We shall have, therefore, equations such as the following:

$$\frac{dc_{\mu-1}}{dt} = 0,$$

whence from (4)

$$k'_{\mu-1} e_{\mu-1} - K_{\mu-1} c_{\mu-1} n = 0. \tag{6}$$

(*b*) *Expression of a condition for cell division.* At this stage we have to consider the relation between the total amount of enzyme and the number of cells, n. Some of the enzymes, doubtless, play a more vital role in the cell than others. It is by no means difficult to imagine an enzyme, or a group of enzymes, which must be built

up to some necessary minimum before the cell is ready to divide. Let the subscript μ apply to an enzyme upon the full complement of which the division waits. Then there will be direct proportionality between n and e_μ. This being so,

$$n = \beta e_\mu, \tag{7}$$

where β is a constant.

(*c*) *Proportions of enzymes.* From (6) and (7),

$$k'_{\mu-1} e_{\mu-1} - K_{\mu-1} c_{\mu-1} \beta e_\mu = 0,$$

whence

$$c_{\mu-1} = \frac{k'_{\mu-1}}{\beta K_{\mu-1}} \frac{e_{\mu-1}}{e_\mu}. \tag{8}$$

Integrating (3),

$$e_{\mu-1} = (e_{\mu-1})_0 e^{k_{\mu-1} t}, \tag{9}$$

where $(e_{\mu-1})_0$ is the amount present at the time zero.

Inserting (8) and (9) in (5),

$$\frac{de_\mu}{dt} = \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1}} \frac{e_{\mu-1}}{e_\mu} e_\mu \tag{10}$$

$$= \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1}} e_{\mu-1},$$

$$\frac{de_\mu}{dt} = \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1}} (e_{\mu-1})_0 e^{k_{\mu-1} t}. \tag{10a}$$

Integration of (10a) gives

$$\frac{e_\mu - (e_\mu)_0}{e_{\mu-1} - (e_{\mu-1})_0} = \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1} k_{\mu-1}}. \tag{11}$$

(*d*) *Logarithmic growth phase.* When growth has proceeded for some time so that the amount of new substance outweighs that present at time zero, the left-hand side of (11) approaches more and more closely to $e_\mu / e_{\mu-1}$. In other words, in the normal steady state the ratio of the amounts of the two enzymes is constant:

$$\frac{e_\mu}{e_{\mu-1}} = \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1} k_{\mu-1}} = \gamma. \tag{12}$$

It should be observed, however, that although the ratio is constant during the period of logarithmic growth (i.e. during this period the proportions of the two enzymes do not change), nevertheless γ changes with the value of the constants in (12). Any influence which modifies the values of these will cause a change in the balance of the enzymes. This will prove to be of importance in the discussion of adaptive processes.

In equation (3) the enzyme is assumed saturated with respect to

its substrate. Had this not been postulated, the concentration $c_{\mu-2}$ would have had to be derived from an earlier equation of the series. This process might have had to be repeated a number of times. Eventually, however, one of the enzymes is encountered for which the introduction of a c term is unnecessary. In the last resort this must apply to the enzymes responsible for the utilization of the primary nutrient substances provided by the medium. Equation (12) may therefore be regarded as generally applicable during the logarithmic phase.

Equation (9) states

$$e_{\mu-1} = (e_{\mu-1})_0 e^{k_{\mu-1}t},$$

and equation (10) may be written:

$$\frac{1}{e_\mu}\frac{de_\mu}{dt} = \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1}}\frac{e_{\mu-1}}{e_\mu}. \tag{13}$$

After growth has been proceeding for some time

$$\frac{e_\mu}{e_{\mu-1}} = \frac{k_\mu k'_{\mu-1}}{\beta K_{\mu-1} k_{\mu-1}}, \quad \text{from (12).}$$

Therefore

$$\frac{1}{e_\mu}\frac{de_\mu}{dt} = k_{\mu-1}$$

or

$$e_\mu = (e_\mu)_0 e^{k_{\mu-1}t}, \tag{14}$$

and if we choose formally to write

$$e_\mu = (e_\mu)_0 e^{\chi_\mu t},$$

then

$$\chi_\mu = k_{\mu-1}.$$

In other words the exponential constants for the enzymes are equal.

This is only true so long as we are able to replace $e_{\mu-1}/e_\mu$ in (10) by the value γ which it attains after continued growth. If $(e_\mu)_0$ and $(e_{\mu-1})_0$ in (11) had *arbitrary* values, it would be some time before (9) and (14) were simultaneously applicable.

If, however, a culture has been maintained in a given medium for some time, then on serial subculture during the logarithmic phase, the value of $(e_\mu)_0/(e_{\mu-1})_0$ for the new period of growth is the same as $(e_\mu)_\infty/(e_{\mu-1})_\infty$ for the old, and (9) and (14) apply from the beginning, since now $\dfrac{e_\mu-(e_\mu)_0}{e_{\mu-1}-(e_{\mu-1})_0} = \gamma$ throughout.

Logarithmic growth cannot be expected if the value of γ is seriously different in the new medium from what it was in the old: nor will this mode of growth set in immediately if the ratio of the enzymes

at the time of subculture differs from that established during the previous logarithmic phase in the same medium. The former case will be considered later in connexion with adaptation. The latter is clearly related to the lag phase.

(*e*) *Simple model of lag phase depending upon enzyme decay.* During the stationary phase some of the enzymes decay. For the purposes of this discussion we shall take the case where one particular enzyme has decayed much more than the others. Let this one be the $(\mu-1)$th. Since the $(\mu-1)$th is present in much less than normal amount, the functioning of the μth will be affected, and division will be delayed. This delay will constitute one form of lag, which has something in common with the lags actually observed.

In the calculation which follows it will be assumed that the decayed enzyme begins to recover at the normal rate as soon as transfer to the new medium occurs, no other disorganization of the reaction sequence having occurred. This assumption is not believed to be correct, but it will be useful to have before us the consequences to which it leads.

The growth equations are given by (3), (4), and (5). Since we shall be concerned with the period preceding the first cell division, n will be n_0 throughout, n_0 being the number of cells in the inoculum taken from the old medium and transferred to the new at time zero. To simplify notation $(\mu-1)$ will now be written as 1 and μ as 2.

We have then:

$$de_1/dt = k_1 e_1, \tag{15}$$

$$n_0 dc_1/dt = k_1' e_1 - K_1 c_1 n_0, \tag{16}$$

$$de_2/dt = k_2 e_2 c_1. \tag{17}$$

(15) gives $e_1 = (e_1)_0 e^{k_1 t}$,

so that (16) becomes†

$$n_0 \frac{dc_1}{dt} + K_1 n_0 c_1 = k_1 (e_1)_0 e^{k_1 t}.$$

The solution of this last equation is

$$c_1 = (c_1)_0 e^{-K_1 t} + \frac{k_1'(e_1)_0}{n_0(k_1+K_1)} \{e^{k_1 t} - e^{-K_1 t}\}, \tag{18a}$$

where $(c_1)_0$ is the value of c_1 at $t = 0$.

† K_1, the constant governing the rate of loss of intermediate by diffusion, will in fact vary slightly as the lag ends and the cells begin to grow larger. An average value may, however, be assumed for the present purpose without serious error.

(17) now becomes

$$\frac{de_2}{dt} = k_2 e_2\left[(c_1)_0 e^{-K_1 t} + \frac{k_1'(e_1)_0}{n_0(k_1+K_1)}\{e^{k_1 t} - e^{-K_1 t}\}\right],$$

which by integration yields

$$\ln\frac{e_2}{(e_2)_0} = \frac{k_2(c_1)_0}{K_1}(1-e^{-K_1 t}) + \frac{k_2 k_1'(e_1)_0}{n_0(k_1+K_1)}\left\{\frac{e^{k_1 t}-1}{k_1} + \frac{e^{-K_1 t}-1}{K_1}\right\}, \quad (18\,b)$$

where $(e_2)_0$ is the amount of e_2 at $t = 0$.

If the inoculum comes from an old culture, the intermediates will have diffused out of the cells and $(c_1)_0$ will be nearly zero. In this case the first term on the right-hand side of (18 b) may be neglected.

According to our postulated conditions $(e_1)_0$ is small compared with the normal complement, while $(e_2)_0$ starts at the standard value. The lag will end when $(e_2)_0$ has increased to $2(e_2)_0$. Up to this point n will remain constant at n_0. $(e_1)_0$ being small, t must be considerable, so that (18 b) may be written in the approximate form

$$\ln\frac{e_2}{(e_2)_0} = \frac{k_2 k_1'(e_1)_0}{k_1 n_0(k_1+K_1)} e^{k_1 t},$$

or, if L is the lag,

$$\ln 2 = \frac{k_2 k_1'(e_1)_0}{k_1 n_0(k_1+K_1)} e^{k_1 L}.$$

Since from (7) and (12) respectively

$$n_0 = \beta(e_2)_0 \quad \text{and} \quad (e_2)_0/(e_1)_{0\,\text{normal}} = k_2 k_1'/\beta K_1 k_1,$$

we obtain

$$\ln 2 = \left\{\frac{K_1}{(k_1+K_1)}\frac{(e_1)_0}{(e_1)_{0\,\text{normal}}}\right\} e^{k_1 L}.$$

If the age of the cells used for the inoculum be denoted by S, the simplest expression for $(e_1)_0$ is

$$(e_1)_0 = (e_1)_{0\,\text{normal}}\, e^{-\lambda S},$$

λ being a decay constant.

The last equation then becomes

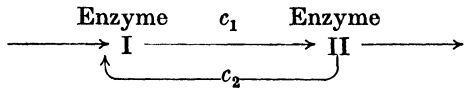
$$k_1 L = \lambda S + \ln\left\{\frac{0{\cdot}693(k_1+K_1)}{K_1}\right\}. \quad (19)$$

According to this the lag would increase linearly with the age of the cells. In practice such behaviour may be observed over a certain range. Usually, however, the lag increases rather rapidly at first and

then more slowly.† There was in fact no imperative necessity to assume a simple exponential decay law for the enzyme: more complex laws are expressible by the introduction of a variable λ. If, for example, the enzyme decayed not according to the assumed first order law, but, as is conceivable, according to a second order law, then λ would diminish with decreasing e_1 and the lag would increase with age at a rate which decreased as time went on.

3. Cyclically linked enzyme model

One very important class of linked reactions is typified by the following simple system:



which contains the essentials of the problem. It is supposed that one of the later enzymes of the sequence produces a diffusible intermediate which plays a necessary part in the functioning of one of the earlier enzymes—acting, for example, in conjunction with another substrate provided by the external medium. The synthesis and operation of enzyme I in the diagram is here supposed to require the intermediate from II: and the set of equations which now applies is taken to be

$$de_1/dt = k_1 c_2 e_1, \tag{1}$$

$$ndc_1/dt = k_1' c_2 e_1 - K_1 c_1 n, \tag{2}$$

$$de_2/dt = k_2 c_1 e_2, \tag{3}$$

$$ndc_2/dt = k_2' c_1 e_2 - K_2 c_2 n. \tag{4}$$

These equations differ from the set (15), (16), and (17) on p. 79 by the addition of the last, and by the introduction of the factor c_2 into (1) and (2). Enzyme I may, of course, require substrates other than that returned from enzyme II, but its rate of synthesis and action is assumed independent of their concentration.

† In the experiment of Cole and James referred to in the footnote on p. 54 the decay of the enzymes was in fact approximately exponential. The values of λ could be directly determined and employed to calculate the rate of increase of L, on the assumption that k_1 had the normal value derived from growth experiments. The result, as stated, was that the predicted rate of increase of the lag was not nearly so great initially as that actually observed. Expressed in another way, the value of k_1 which would have to be assumed to give agreement would be much smaller than that derived from the logarithmic growth rate. This means that, if the enzyme has decayed its initial recovery is slower than that assumed in this model: in other words the lag depends upon other disturbances also (see the following section).

The most interesting consequence of these new equations is in connexion with the lag phase. In the last section we were concerned with the development of lag caused by the decay of enzymes. We may now consider more closely the lag which might arise as a result of disturbances, by loss during the stationary phase, of the steady concentrations of the various diffusible intermediates.

From the equation (18a) of the last section it is obvious that if $(c_1)_0$ is zero, that is if all the intermediate has been dispersed from the cells before they are transferred to the new medium, then

$$c_1 = \frac{k_1'(e_1)_0}{n_0(k_1+K_1)}\{e^{k_1 t}-e^{-K_1 t}\},$$

and the rate at which the new concentration is built up depends, apart from the constants k_1 and K_1, only on the initial amount of the *enzyme*. The regeneration of c_1 is independent of $(c_1)_0$. Therefore no very long lag can be expected unless there has been an appreciable decay of the enzymes themselves.

The matter is quite different according to the new set of equations (1) and (4).

During the lag n remains constant at n_0. Moreover, since we are considering dispersal of intermediates rather than decay of enzymes, we may take, with a good degree of approximation, $e_1 = (e_1)_0$ and $e_2 = (e_2)_0$ during most of the lag.

Therefore (2) and (4) become:

$$\frac{dc_1}{dt} = \frac{k_1'}{n_0}(e_1)_0 c_2 - K_1 c_1, \tag{5}$$

$$\frac{dc_2}{dt} = \frac{k_2'}{n_0}(e_2)_0 c_1 - K_2 c_2. \tag{6}$$

The solution of these equations gives

$$c_1 = Ae^{\alpha t}+Be^{\beta t},$$

where α and β are the roots of the equation

$$m^2+(K_1+K_2)m+K_1K_2-\frac{k_1'}{n_0}(e_1)_0\frac{k_2'}{n_0}(e_2)_0 = 0.$$

When $t = 0$, $c_1 = (c_1)_0$ and $c_2 = (c_2)_0$, whence

$$c_1 = \frac{(c_2)_0(k_1'/n_0)(e_1)_0-(c_1)_0(K_1+\beta)}{\alpha-\beta}e^{\alpha t}+\frac{(c_2)_0(k_1'/n_0)(e_1)_0-(c_1)_0(K_1+\alpha)}{\beta-\alpha}e^{\beta t},$$

with a corresponding expression for c_2.

The important fact here is that the factors multiplying the exponential terms are both linear functions of $(c_1)_0$ and $(c_2)_0$, not merely, as before, of $(e_1)_0$ or $(e_2)_0$. Thus if $(c_1)_0$ and $(c_2)_0$ were both zero, c_1 and c_2 would remain zero for all time. The essential result is more clearly seen if we introduce the simplification that

$$K_1 = K_2 \quad \text{and} \quad k_1'(e_1)_0/n_0 = k_2'(e_2)_0/n_0.$$

We have then

$$c_1 = \frac{(c_1)_0+(c_2)_0}{2} e^{(k_1'(e_1)_0/n_0-K_1)t} + \frac{(c_1)_0-(c_2)_0}{2} e^{-(k_1'(e_1)_0/n_0+K_1)t},$$

$$c_2 = \frac{(c_1)_0+(c_2)_0}{2} e^{(k_1'(e_1)_0/n_0-K_1)t} - \frac{(c_1)_0-(c_2)_0}{2} e^{-(k_1'(e_1)_0/n_0+K_1)t}.$$

These last equations differ from equation (18 a) of the last section in that the rate of restoration of c_1 and c_2 depends very much upon $(c_1)_0$ and $(c_2)_0$. Both of these appear in the multipliers of the exponential terms. With the initial values zero there is no recovery. If the initial values are small enough, then recovery may require a time which can be made as great as we please. Thus any value of the lag, up to infinity, could be accounted for in terms of the loss of intermediates. The cyclical mechanism postulated here is not at all improbable in itself. The consequences of its operation are such that recovery from a serious disturbance of the steady state is very much more difficult than it would be with a non-cyclical mechanism.† Naturally the example which has just been discussed has been simplified in the most extreme degree possible, but one can see quite clearly that the same kind of result must apply to more elaborately coupled systems. There is, of course, an analogy between the slow and difficult recovery predicted for the chemical systems constituting the cell economy and the extreme tardiness with which complex interlocking national economies can right themselves after the dislocations of supply resulting from wars.

From quite a different point of view it is interesting to note that according to equations (2) and (4) there is no necessary steady concentration of the intermediates during actual growth. If we equate

† The results quoted in the footnotes on pp. 54 and 81 show that in fact the lag develops more quickly than would be accounted for by enzyme decay, or by the dispersal of a single intermediate. They therefore indicate that factors of the kind discussed in this section are at work. The reservation must, however, be made that the experiments quoted are only an initial approach to what needs much more thorough investigation.

dc_1/dt and dc_2/dt to zero for a steady state, then we only obtain a value for the *ratio* c_1/c_2. This result differs from that given by the expression (16) of the last section which, equated to zero, defines c. Thus, as far as the present equations go, c_1 and c_2 could both continue to increase indefinitely, each stimulating the further increase of the other. But, in fact, one or other must reach such a value that the corresponding enzyme is saturated with substrate. Thus a stationary state will be imposed by conditions other than equations (2) and (4). It will correspond to a condition where one of the enzymes is *just working at its maximum possible rate.* This result may be very significant in connexion with the considerations advanced on pp. 120 and 140.

As far as the considerations of this section and of the last in respect of the lag go, we may summarize by saying that the decay of enzymes may give as long a lag as we need to account for: dispersal of intermediates will also do this if we postulate some form of cyclic mechanism in the complete reaction sequence.

Further experimental studies of the development of the later stages of the lag-age relation, with parallel investigations of changing enzyme activities, will probably give interesting results from the point of view of this section.†

4. Rate determining steps : steady states

It has sometimes been the custom to speak of a 'master reaction' which is rate-determining for a whole chain of consecutive processes, such as occur in cells. The reserve with which such a conception must be treated has also been emphasized.‡ The velocity of a series of consecutive reactions is certainly not governed in general by that of the slowest. This is easily seen by considering a simple example, namely a series of consecutive first order reactions, familiar in connexion with radioactive disintegrations:

$$A \longrightarrow B \longrightarrow C.$$

From the equations

$$-\frac{dA}{dt} = k_A A, \qquad \frac{dB}{dt} = k_A A - k_B B, \qquad \frac{dC}{dt} = k_B B,$$

one obtains $$C = A_0\left\{1 - \frac{1}{k_A - k_B}(k_A e^{-k_B t} - k_B e^{-k_A t})\right\},$$

† See footnotes on pp. 54, 81, and 83.

‡ A. C. Burton, *J. Cell Comp. Physiol.*, 1936, **9**, 1.

from which it is obvious that the rate of production of C depends both upon k_A and upon k_B and not upon either of them separately.

On the other hand, if k_B can be assumed to be very much greater than k_A, the value of C approximates closely to the form

$$C = A_0\left\{1+\frac{1}{k_B}(0-k_B e^{-k_A t})\right\}$$
$$= A_0(1-e^{-k_A t}) = A_0 - A.$$

That is to say, provided that k_B can be regarded as very great compared with k_A, then the rate of formation of C can be taken as independent of its precise value. Examples are common enough in chemistry where one of the velocity constants is so great that its exact value need not be taken into consideration. But this is only true in special circumstances. It is not enough merely for one reaction to be slower than the others for it to become a rate-determining step.

It will now be useful to consider how far the simple model which was discussed in the last section will be subject to a master-reaction or rate-determining step. For this purpose the equations of section 2 will be rewritten in a rather more general form:

$$\frac{de_{\mu-1}}{dt} = k_{\mu-1}\, e_{\mu-1} f_{\mu-1}(c_{\mu-2}), \tag{1}$$

$$n\frac{dc_{\mu-1}}{dt} = k'_{\mu-1}\, e_{\mu-1} f_{\mu-1}(c_{\mu-1}) - K_{\mu-1}\, c_{\mu-1}\, n - k_\mu\, e_\mu f_\mu(c_{\mu-1}) = 0, \tag{2}$$

$$\frac{de_\mu}{dt} = k_\mu\, e_\mu f_\mu(c_{\mu-1}). \tag{3}$$

These differ from the previous set in two ways: first, the influence of the substrate concentration on the speed of the enzyme reaction is expressed by the unspecified function f:† secondly, in (2) the loss of intermediate by diffusion is no longer assumed to play the principal part in balancing its production. The third term on the right of (2) represents the rate of consumption of the intermediate by the following enzyme of the sequence. Otherwise the assumptions and symbols are the same as those of the previous sections.

† It will be convenient to express the function f in such a way that it becomes unity when the enzyme is saturated and the rate independent of substrate: this saves the introduction of extra proportionality constants, but is otherwise trivial.

Introducing certain abbreviations, we may rewrite (2)

$$a\frac{de_{\mu-1}}{dt} - Zn - \frac{de_\mu}{dt} = 0, \tag{4}$$

where $a = k'_{\mu-1}/k_{\mu-1}$ and $Z = K_{\mu-1}c_{\mu-1}$.

Integration gives

$$a\{(e_{\mu-1}) - (e_{\mu-1})_0\} - Z\int n\,dt - \{(e_\mu) - (e_\mu)_0\} = 0. \tag{5}$$

In the present example let the $(\mu-1)$th enzyme be that which bears a constant ratio to n. Then

$$e_{\mu-1} = (e_{\mu-1})_0 e^{k_{\mu-1}t} \quad \text{and} \quad n = n_0 e^{k_{\mu-1}t}.$$

With $Z' = Z/k_{\mu-1}$, (5) therefore becomes

$$e_\mu - (e_\mu)_0 = \{a(e_{\mu-1})_0 - Z'n_0\}e^{k_{\mu-1}t} - a(e_{\mu-1})_0 + Z'n_0$$

which, when the actual values considerably outweigh the initial values, reduces to

$$e_\mu = \{a(e_{\mu-1})_0 - Z'n_0\}e^{k_{\mu-1}t}. \tag{6}$$

This is required by the equation (2) which expresses the possibility of a steady state. But according to (3)

$$\frac{1}{e_\mu}\frac{de_\mu}{dt} = k_\mu f_\mu(c_{\mu-1}),$$

whence

$$e_\mu = (e_\mu)_0 e^{k_\mu f_\mu(c_{\mu-1})t}.$$

Thus the condition for a steady state is that

$$k_\mu f_\mu(c_{\mu-1}) = k_{\mu-1}.$$

This is attainable in one of two ways. If the enzyme is saturated with substrate, so that f_μ is unity, then a stable steady state is only possible under the condition that $k_\mu = k_{\mu-1}$.

On the other hand, if the enzyme is not saturated, and its rate is responsive to changes in $c_{\mu-1}$, then $c_{\mu-1}$ will adjust itself to such a value that the steady state is established.

Thus the relations of the $(\mu-1)$th and the μth enzyme may be envisaged as follows. If the second is inherently capable of much more rapid growth than the first, then it will keep the substrate concentration near zero and its own expansion can only occur at an absolute rate governed by the material which the first passes on to it. Its own formation must settle down to bear a constant ratio to that of the first, determined by a purely stoichiometric factor. From the point of view of equation (2) this is not a steady state, but from

the point of view of cell economy it maintains constancy of composition. If the second enzyme grows more slowly than the first, even when saturated with intermediate, no steady state is possible and it will be eliminated on further growth. The most important case, however, is that where the second enzyme grows more slowly than the first at very small values of c, and faster for high values. Then as increase in bacterial mass occurs, the first may tend to increase relatively to the second: this increases c, which in turn causes more rapid growth of the second, so that a steady state is established and can be maintained indefinitely, as long as the conditions are kept constant.

Having considered the relations of the $(\mu-1)$th and the μth enzymes, we must now give some attention to those of the $(\mu-1)$th to its precursors.

If all the enzymes of the sequence are linked in the way described, and all are responsive to the concentrations of the intermediate substrates, then just as the μth comes to be formed in a constant ratio to the $(\mu-1)$th, so the $(\mu-1)$th does to the $(\mu-2)$th and so on back to the first of the series. The rate of formation of the first will, of course, be a function of external concentrations of foodstuffs. Thus the whole set of enzymes will tend to settle down to constant proportions. If at any stage of the sequence there is an enzyme whose inherent rate is so great that it consumes all the substrate which can be supplied, then this only makes its proportion still more dependent upon the previous members of the series. If, on the other hand, there were one whose rate was so small that no increase in its substrate concentration could cause it to keep pace, then it, and all subsequent members of the series, would be eliminated: and the postulated sequence would not play any part in bacterial processes.

In a certain sense it might be permissible to regard the first step of the sort of sequence described above as a master reaction: though it by no means determines the proportions of the individual enzymes. However, a further possibility must be borne in mind, and this suggests even greater reserve about the use of the term. Hitherto, except in section 3, we have spoken of the enzyme processes as though they constituted one single sequence. Discussions based upon this idea may serve as very useful general guides, but they probably represent an over-simplification. What, in principle, seems quite possible is precisely the situation considered in section 3, namely

that the product of one of the later enzymes of a sequence should itself participate in conjunction with other substrates in the operation of one of the earlier enzymes. Some of the consequences of such a 'feed-back' mechanism have already been indicated and will not be discussed at this juncture: though the possibility of this complication must always be borne in mind. Another possible complication is the existence of separate sequences with different starting-points, involving, for example, different components of the medium, the two series converging at a certain stage. Discussion of this must be deferred.

5. Some applications of the enzyme model

(*a*) *Enzyme proportions and activities in cells.* In an established and prolonged logarithmic growth phase, if external conditions remain constant, the enzymes must settle down to constant ratios, and their activities should reach and retain steady values. But, under normal conditions of growth, the logarithmic phase is not indefinitely prolonged, and the external conditions change. Foodstuff becomes exhausted, toxic products accumulate, and the pH varies. The values of the individual constants in the equations of the two previous sections are themselves changing, so that the steady state is not really reached, and the cells tend always to be moving from one state of equilibrium to another. This is particularly true when large inocula of old cultures are subcultured into new medium not capable of supporting very heavy growth: before the true steady-state proportions of cell material, seriously disturbed by decay during the stationary phase, are re-established in logarithmic growth, the disturbances due to the approach to a new stationary phase begin to manifest themselves. In such circumstances it is hard for the culture ever to become truly stabilized. The proper conditions for stabilization are repeated subculture into fresh medium of inocula never allowed to age.

This matter will be further discussed later on in the present chapter.

(*b*) *Adaptive phenomena.* When cells which have reached a stable condition in one medium are transferred to another, the constants of all the separate processes are likely to possess different values. A new stable state has to be established. It will be shown that this readjustment is, in all probability, closely connected with the adaptive phenomena shown by bacteria when they are grown in changed

media. The whole matter will be discussed more fully in a later chapter, and a few preliminary observations are all that will be made here.

Suppose under the changed conditions the value of the constant k_μ has a smaller value. This would tend at first to cause an increase in the amount of the $(\mu-1)$th enzyme relatively to the amount of the μth. But this increase will cause a rise in $c_{\mu-1}$, which can in turn stimulate more growth of the μth. In such a case adaptation would occur and would be accompanied by a change in one or more of the intermediate concentrations. In the case where the steady state depended upon the absolute equality of $k_{\mu-1}$ and k_μ (see p. 86), this automatic regulation by way of the intermediate concentration could not occur: any adaptation would involve an actual change in the value of k_μ. This could only come about as a result of a modification in the texture of the enzyme material itself. We shall see later that it may be necessary to postulate two kinds of adaptive change: one dependent upon mere changed proportions of enzyme material, the other associated with actual structural modifications.

(*c*) *Drug action.* Drugs may modify specifically any of the constants of the growth equations. If the rate of the primary foodstuff utilization is cut down, no automatic adaptive response can be predicted from the foregoing considerations. If, however, the principal influence is exerted upon the operation of the μth enzyme of the sequence, then an expansion of the $(\mu-1)$th enzyme can give an increased intermediate concentration which can counteract the inhibition due to the drug. Partial or complete self-immunization of the cells may then occur. This matter will be discussed more fully in a subsequent chapter.

6. Experimental studies of enzyme activities

The principle of these experiments is that a culture is grown to a given stage, separated from its medium, usually by centrifuging, re-suspended in a suitable liquid such as a dilute salt solution or a buffer, and tested for its activity in promoting some specific chemical reaction, such as oxidation, deamination, decarboxylation, proteolysis, and so on.

The usual finding is that the enzymatic activity, for a standard mass or number of cells, varies with the age of the culture from which they were taken, starting low, passing through a maximum during

the logarithmic phase, and then declining. Typical examples are dehydrogenases† of *Bact. coli*, deaminases of *Clostridium Welchii*,‡ amino-acid decarboxylases of *Bact. coli*,§ and the catalase of *Bact. lactis aerogenes*.‖

Now it is necessary to draw a distinction between amount of enzyme substance and enzyme activity. The functioning of the enzyme in a specific reaction frequently demands the co-operation of a diffusible co-enzyme. When this is so, the activity is determined not merely by the state of the cell material itself but by what has been built up in the medium. As we have seen in the discussion of the early lag phenomenon (p. 50), loss of active substances by diffusion may delay growth: similar losses may delay the attainment of optimum enzyme activities. Optimum growth and optimum reactivity in respect of a particular chemical change induced by the cell enzymes will not invariably coincide, and, for obvious reasons, growth would be expected to be the first under way. Woods and Trim‡ showed that in the action of *Clostridium Welchii* on serine in a medium where growth could occur, the enzyme activity lagged behind growth in the earlier stages. That this was connected with the loss of a co-enzyme by diffusion was shown by the fact that dilution of the cell suspension caused a very much more than linear fall in its activity—which was reduced practically to zero when the suspension was diluted to 1/10. Dilution of *Bact. coli* suspensions causes an analogous drop in the activity of their lactic acid dehydrogenase,¶ which again suggests a diffusible co-enzyme. Another observation of this kind has been made with proteinase in *Clostridium histolyticum*.†† A strain of this organism produces an extracellular proteinase which can be demonstrated in the filtrate and which is activated by cysteine. On subculture the proportion of proteolytic enzymes has been observed to change, and cultures have been obtained giving both intra-cellular and extra-cellular proteinases. There is, it may be remarked, evidence that the passage into the medium of the active substances is not due to destruction of dead

† W. R.Wooldridge, R. Knox, and V. Glass, *Biochem. J.*, 1936, **30**, 926; W. R. Wooldridge and V. Glass, ibid., 1937, **31**, 526.

‡ D. D. Woods and A. R. Trim, ibid., 1942, **36**, 501.

§ E. F. Gale, ibid., 1940, **34**, 392.

‖ See Fig. 17.

¶ J. Yudkin, *Biochem. J.*, 1937, **31**, 865.

†† W.-E. van Heyningen, ibid., 1940, **34**, 1540; L. Weil, W. Kocholaty and L. D. Smith, ibid., 1939, **33**, 893.

cells, but occurs from the living bacteria.† The decay of the activity of the enzyme responsible for the deamination of serine by washed suspensions of *Bact. coli* was found by Gale and Stephenson‡ to be partly due to the loss by diffusion from the cells of some substance acting as a co-enzyme.

These and other observations of similar kind show that the activity of the cell in promoting some specific chemical reaction is often a function, not merely of the amount of enzyme substance, but of the concentration of auxiliary substances in the cell and the surrounding medium. One sees that in general this will cause the development of activity to lag behind the formation of substance, except in so far as the loss of diffusible material is prevented by addition of filtrate from older cultures to the medium. The question now arises as to the place of these phenomena in the picture of cell development which has been considered in the previous sections. In this, amount of enzyme was taken to mean amount of the basic material substance of the enzyme. The examples which have just been referred to, however, involve the consideration of loss of diffusible substances from the cell. For theoretical treatment, therefore, a method similar to that outlined in connexion with 'early lag' in III. 5 is indicated. Formally, however, we could apply the method of the previous sections of the present chapter with the introduction of a variable value of $K_{\mu-1}$. Suppose the intermediate formed by the $(\mu-1)$th enzyme is readily lost by diffusion into the external medium. Then the rate of loss will be a function, not only of the concentration in the cell, but also of that in the medium outside. As growth proceeds and the *external* concentration increases, we have what is equivalent to a fall in $K_{\mu-1}$. The loss now drops, $c_{\mu-1}$ rises, and the activity of the μth enzyme increases.

Thus a complete theoretical treatment involves a combination of that of the previous sections with that of III. 5. There would be certain difficulties about this, and in some ways it would be better to plan the experimental approach somewhat differently, making, by the appropriate methods, separate investigations of the changes in enzyme activity for the distinct parts of the growth cycle.

First, the development of enzyme activity during the lag phase

† L. Weil and W. Kocholaty, ibid., 1939, **31**, 1255; A. Janke, *Z. Bakt.*, 1939, **144**, 122.

‡ E. F. Gale and M. Stephenson, *Biochem. J.*, 1938, **32**, 392.

of resting cells, before the onset of growth, could be studied in relation to number of cells and concentration of added filtrate from older cultures. Secondly, the decay of activity of cells during the stationary phase following growth could be followed and studied in relation to the nature of the medium (whether the used growth medium or a new medium to which the cells are transferred after washing). In this way the relative importance in the decline of activity of pH, simple loss by diffusion of accumulated intermediates, and so on, could be assessed.

The course of events during the logarithmic phase itself is rather difficult to deal with. In the first place, the duration of this phase is usually rather short. Secondly, the delay in development of activity due to loss by diffusion and that due to retarded establishment of the correct material balance in the cell are rather difficult to distinguish. This latter point will now be elaborated a little. The discussions of earlier sections have shown that a steady ratio of enzymes, in the material sense, can only be expected when the amount of new growth outweighs considerably the substance of the inoculum—unless the latter happened to be taken immediately from a culture just in the equilibrated condition itself. Ordinarily the inoculum will come from a culture in which the enzymes will have decayed in varying degrees: their proportions will not correspond to those of the steady state. This state cannot be restored until some considerable amount of growth has occurred. Strictly speaking, the logarithmic phase itself will not be fully established until the enzyme ratios have achieved equilibrium, but in practice it is probably nearly reached as soon as the more essential growth enzymes are in balance. Other less essential enzymes may not be in balance until what is experimentally indistinguishable from logarithmic growth has been in operation for a time. If the logarithmic phase is prolonged all must come into balance: except that when it is artificially prolonged by continual transfer to new medium, the complication of the dilution of diffusible growth factors is introduced afresh each time.

7. Cyclical changes in bacterial properties

In the light of the foregoing discussion it is convenient to consider one possible cause of a phenomenon which has from time to time excited comment and curiosity, and which at first sight seems like a manifestation of an arbitrary quality of living matter. When

bacteria are subjected to serial subculture in a given medium, they sometimes give the impression of passing through rather irregular cyclical variations. For example, after growing for a number of subcultures in the form of a suspension of well-separated cells, a pronounced tendency to form clumps may develop, the culture showing under the microscope the appearance of matted groups of cells. As the subculturing process is continued, this habit is lost again. Or again, the enzyme activity of cells in respect of a particular enzyme reaction may, even at a standard age of the culture, show a certain fluctuation from one serial subculture number to the next.

Now the normal practice in making serial subcultures is to effect a transfer at standard intervals, for example, every 24 hours. Let us now consider the course of events starting from a given serial number. In general, at the moment of transfer the cells will have shown some decline, even if a small one, from the maximum enzyme activities of the constituents: and this decline, since the decay constants for the various enzymes will be specific properties of the individuals, will have gone on to varying degrees in different parts of the cell substance. During the next phase of growth the enzyme ratios will tend to return to the standard equilibrium values, and will reach them if the logarithmic multiplication continues long enough. Since, however, the logarithmic phase is of finite extent, the original proportions will be only approximately and not exactly re-established. If, for example, the amount of bacterial substance increased tenfold during the growth, then about 90 per cent. of the substance should be of the standard composition and 10 per cent. might still depart from it. (This assumes for simplicity what is probably not correct, namely, that no repair of the material or rectification of proportions occurs during the lag phase: but the general argument is not affected by the neglect of this consideration at the present moment.) An additional factor now comes in: if considerable decay of enzyme substance has occurred before our first subculture, the lag will be correspondingly great. For this very reason, the grown culture will be younger at the 24-hour stage than it would have been had the lag been longer. Thus we shall automatically start the next subculture with more nearly the standard enzyme ratios, and during the subsequent growth still further return to normality occurs. But, since the lag this time has been shorter, there is more time for the culture to age before the next 24-hour transfer, so that greater opportunity

for relapse is provided. Once again, the relapse differs in seriousness with different enzymes. We thus have an alternation of decay and restitution going on with a periodicity which not only is irregular but possesses a different kind of irregularity for various parts of the cell. The result is a complicated, though roughly cyclical, variation in certain enzyme proportions, and, correspondingly, one may well believe, in certain specific properties of the cells. The irregularities will be the more marked the shorter the logarithmic growth phase: because with a very long logarithmic phase, one would always have complete restitution of the true equilibrium proportions. If the latter are never given a real opportunity to re-establish themselves, then the cyclical variations referred to will not, strictly speaking, be closed ones, since it is unlikely that the various periodicities created by the subculturing procedure stand in any precise relation to one another. In such an event the cell would show irregular fluctuations in properties with secular trends towards ever new ones. The latter, fortunately, are slow enough, or unimportant enough, not to remove bacteriology from the realms of quantitative science. The former may well account for the many references in the descriptive literature to 'Life cycles'. Since the properties showing the fluctuations which we have been discussing include those which determine morphology, the erratic appearance of short rods, filaments, and so on can be understood. The assumption that these represent an ordered sequence of forms through which each individual passes at the appropriate stage, as with certain more complex organisms, seems not to be necessary.

V

THE ACTION OF DRUGS ON CELLS AND THE ADAPTATION OF CELLS TO RESIST DRUG ACTION

1. Introduction

THE general problem of the action of drugs on cells belongs to the subject of pharmacology.† In this chapter drug action will only be considered as an instrument for exploring the mode of co-ordination of the various chemical reactions in the cell economy.

The influence of drugs on bacteria is varied. Some act as general protoplasmic poisons, while some have specific effects on particular members of the sequences of enzymatic reactions determining growth. These latter types of action will prove to be of the greater interest for our present purpose.

The action of various inhibitory substances is exerted specifically on the lag, on the rate of growth in the phase of logarithmic increase, on the cell division probability, or even on the total bacterial population supportable by the medium. Since these effects have not usually been very clearly distinguished, there is some difficulty in interpreting many of the results recorded in the literature. Another distinction, upon which a good deal of stress has, however, been laid is that between bacteriostatic and bactericidal action. Since all bacteria die sooner or later if they are not allowed to multiply, any agent which retards growth for a long enough time will prove to be bactericidal. In the last resort, therefore, the distinction is one of degree rather than one of kind: nevertheless for many practical purposes it is of great value.

As regards the relation of drug action and chemical structure, two effects have to be recognized. On the one hand, the partition of the drug between the medium in which the cells grow and the part of the cell where the action is exerted will depend upon the chemical properties of the drug. In particular, the partition coefficient will show more or less regular variations with such changes in the chemical structure of the drug molecule as increasing length of chain,‡ or replacement of one substituent group by another. Many quite important quantitative differences in pharmacological action can be

† See A. J. Clark, *General Pharmacology; Trans. Faraday Soc.*, 1943, Discussion on 'Modes of Drug Action'.

‡ K. H. Meyer, *Trans. Faraday Soc.*, 1937, **33**, 1062.

accounted for in terms of varying phase distributions: and when the actions are compared at equivalent chemical potentials they are seen to be much more nearly equal.† The inhibitory action of substituted phenols on the growth of *Bact. lactis aerogenes* has been shown to be parallel with the solubility of the particular phenol in olive oil, which it is not impossible to imagine as a model of certain parts of the interior of the cell.‡ The inhibitory action of straight chain aliphatic alcohols increases by a constant factor from one member of the homologous series to the next higher one (see § 5).

Partition effects, on the other hand, can hardly be invoked to explain the enormous differences in antibacterial action of such classes of compound as sulphonamides, acridine derivatives, triphenylmethane dyes, potassium tellurite, and penicillin, to mention only a few of the many specifically inhibitory substances. Such differences depend upon the fact that the drugs in question intervene at particular stages of the reaction sequences involved in growth. The evidence for this view is considered more fully in § 2.

In so far as drugs interfere with specific cell reactions, their effects will depend upon the way in which they are taken up by particular parts of the bacterial substance. The concentration of the drug inside the cell in the aqueous medium should be, if not equal to, at least proportional to that in the solution outside, but the amount taken up by the protein or other structures will be related to this concentration by an equation of the same general nature as an adsorption isotherm.§

In special cases the effect of the drug may depend upon its rate of penetration into the cell from outside. With some kinds of cell, and with more complex tissues, penetration may play an all-important part. It varies widely and is highly specific, as the work of Overton and many other workers has shown. Yet this decisive effect of penetration does not seem to have been forced upon the attention of workers with bacterial cells, and certain observations suggest that it is not generally a limiting factor. For example, certain cells of *Propionibacterium pentosaceum* were immunized to the action of sodium fluoride by cultivation in its presence. The immunity might have been ascribed to decreased permeability to the fluoride. Yet

† J. Ferguson, *Proc. Roy. Soc.*, 1939, B, **127**, 387.
‡ A. H. Fogg and R. M. Lodge, *Trans. Faraday Soc.*, 1945, **41**, 359.
§ Cf. remarks on p. 10.

the resistance was not overcome by a 7-hour contact with fluoride solution before the other substances required for the test were added to the system.† Another observation of a somewhat similar kind is the following: if sulphanilamide is left in contact with *Bact. lactis aerogenes* for an hour or two before the carbon source required for growth is added, the effect is no greater than when carbon source and drug are added simultaneously. The quantitative results to be discussed in the next chapter would be rather difficult to interpret in terms of penetration rates, but can be coherently treated on the assumption that penetration is rapid.‡ In some respects the most interesting and striking of all the phenomena relating to the influence of drugs on bacteria is that of adaptation. When cultivated in presence of antibacterial substances at a concentration insufficient entirely to prevent multiplication, the cells frequently acquire the capacity to grow normally under conditions which initially would have permitted only very feeble growth. By successive adaptations at increasing concentrations, strains can be obtained which are immune to hundred of times the amounts of drug which initially would have caused inhibition.

The interest of the phenomenon of adaptation for the purposes of the thesis examined in this book is that quite considerable progress can be made in understanding its nature in terms of kinetic models. Some of the simplest of these will be discussed in detail. As is well known, an alternative explanation of all adaptive changes exists: that, namely, based upon natural selection. It is supposed, according to this, that cells of every degree of drug resistance exist (or can arise by chance) and that those which are best equipped to grow in any given environment outgrow their less well-endowed competitors.

In the treatment of adaptive phenomena we shall first discuss the facts in the light of the hypothesis that the observed changes do indeed represent automatic responses to the new environment: in short, that with altered relative reaction velocities, new enzyme balances are established, and that the properties of the adapted cells are explicable in terms of these. Subsequently, the role of natural selection will be considered. The conclusion which will be reached

† W. P. Wiggert and C. H. Werkman, *Biochem. J*, 1939, **33**, 1061.

‡ Nevertheless more explicit work on penetration of bacterial cells would be of value.

is that while selection may be, and indeed must be, superimposed upon other adaptive mechanisms, there is little profit, with bacterial cells at least, in regarding it as the primary factor governing the adjustment of the economy to resist drugs or to utilize new sources of material.

2. Specific interference with cell reactions by drugs

The work of Fildes, Woods, McIlwain, Gladstone, and others has provided evidence that certain substances may block the whole process of growth by interference at definite stages of the reaction sequence. Fildes† showed that the antibacterial action of mercury compounds may be quantitatively neutralized by compounds containing —SH groups: the addition of glutathione, for example, titrates the toxic effect of mercury in such a way that 2 moles —SH are equivalent to 1 Hg. The conclusion drawn is that the inhibitory action of the mercury depends upon the blocking of sulph-hydryl groups in cell metabolites or enzymes.

An important general idea which seems to have a considerable range of validity is that specific inhibitory actions may be exerted by substances which are structurally related to the normal metabolites of the cell. These substances are then taken up by enzymes in competition with their own normal substrates, but, being useless for further parts of the reaction sequence, block the subsequent steps, and so arrest the growth. Woods‡ suggested that sulphonamides interfere with the metabolism of *p*-amino-benzoic acid in the cell. The utilization rather than the synthesis of this compound is taken to be impeded, since if the latter occurred, addition of *p*-amino-benzoic acid in excess of a certain minimum requirement should give growth independent of sulphonamide concentration. Such a relation is not in fact found, the observed one corresponding to an actual competition of the two substances over the whole range of concentrations.

McIlwain§ has studied inhibition of growth by pyridine-3-sulphonic acid and its amide. These substances can be supposed to act in competition with the nicotinic acid of normal metabolism. The inhibitors can replace the nicotinic acid in the first stage of its use by the cell, but not in later stages. The amino-sulphonic acid

† P. Fildes, *Brit. J. Exp. Path.*, 1940, **21**, 67.
‡ D. D. Woods, ibid., 1940, **21**, 74.
§ H. McIlwain, ibid., 1940, **21**, 25, 136.

analogues of some natural amino-carboxylic acids have an analogous action,† inhibition being observed especially with *Proteus* and with *Staphylococcus*. The antibacterial action is removed by the addition of extra α-amino-carboxylic acid to the medium. When *Staphylococcus* is made independent of amino-carboxylic acids in the medium, as can be done by training, the amino-sulphonic acids no longer exert their inhibitory action. Pantothenic acid analogues‡ may also have antibacterial actions, pantoyltaurine, for example, being antistreptococcal.

Another example of the operation of the same principle is found in the action of indole acrylic acid§ which is thought to inhibit some stage in the synthesis of tryptophan. In contrast with the amino-benzoic acid-sulphonamide antagonism, the addition of traces of tryptophan does not neutralize the inhibitor: thus it is not the actual utilization but the formation of the tryptophan which the indole derivative impedes.

A somewhat similar principle explains the results found by Gladstone‖ on growth in presence of amino acids. Excess of one amino acid may put out of action the centres involved in the synthesis of another related one, so that growth is only possible if that other one is provided in the medium. For example, valine, leucine, or isoleucine added singly to a mixture which supported growth of *Bacillus anthracis* inhibited the growth. Added together they improved and accelerated it.

The various observations referred to are all striking examples of the way in which specific intervention of inhibitory substances occurs in the sequence of cell reactions. As has been emphasized by several writers, these facts and the theory based on them help to constitute a rational basis for the prediction of possible therapeutic agents. It must not, however, be concluded that all drug actions necessarily depend upon structural analogies with normal metabolites: indeed such a statement would not apply to the example of mercury. Again, Wood and Austrian¶ find that with *Staphylococcus aureus* in a synthetic medium, nicotinamide and co-zymase antagonize not only sulphapyridine but quite unrelated drugs also, and from this they

† Idem, ibid., 1940, **21**, 148.
‡ Idem, *Biochem., J.*, 1942, **36**, 417.
§ P. Fildes, *Brit. J. Exp. Path.*, 1941, **22**, 293.
‖ G. P. Gladstone, ibid., 1939, **20**, 189.
¶ W. B. Wood and R. Austrian, *J. Exp. Med.*, 1942, **75**, 383.

conclude that there is no evidence that sulphapyridine interferes necessarily with something chemically related to it.

While we are considering the specific location of drug action it is of interest to note that some antiseptic substances appear to be capable of killing cells before causing any significant inhibition of certain enzymes: namely, the lactic and glyceric dehydrogenases, catalase, peroxidase, and indophenol oxidase of *Neisseria gonorrhoeae*.† There is also a close connexion between this aspect of the subject and the successive deactivations of resting *Bact. coli* described long ago by Quastel and Wooldridge.‡

Another class of observations closely connected with the intervention of drugs at specific stages of the reaction sequence is that relating to the phenomenon of 'cross adaptation'. For example, *Bact. lactis aerogenes* can be trained to resist the action of aminoacridines, which are normally powerful inhibitors. The trained cells also show increased resistance to the antibacterial action of methylene blue, but not to the action of sulphonamides. Training to sulphanilamide gives immunity to sulphaguanidine and vice versa; but sulphonamide training does not give immunity to proflavine.§ *Staphylococci* show cross training to some acridine compounds and to propamidine.‖ Where two drugs act at the same stage of the reaction sequence we may expect cross adaptation: where they act at different stages, none (see also VI. 8).

3. Specific influence on different parts of the growth cycle

From what has been said, we should expect various drugs to have different relative influences on lag, generation time, stationary bacterial population, cell division probability, and so on. This expectation is fully confirmed.

It occasionally appears that cells grow at the normal rate up to a critical concentration of drug above which they fail to grow at all. The explanation seems to be as follows: the drug acts primarily on the lag, but has little effect on the mean generation time. At higher concentrations the drug lengthens the lag to such an extent that the cells all die before growth sets in: if, however, it does set in, as

† M. A. Bucca, *J. Bact.*, 1943, **46**, 151.

‡ J. H. Quastel and W. R. Wooldridge, *Biochem. J.*, 1927, **21**, 148.

§ D. S. Davies, C. N. Hinshelwood, and J. M. Pryce, *Trans. Faraday Soc.*, 1944, **40**, 397.

‖ J. McIntosh and F. R. Selbie, *Brit. J. Exp.* Path., 1943, **24**, 246.

becomes possible at lower drug concentrations, it occurs more or less normally.

In other examples, it must be said, there is a fairly close parallel between the effect of the drug on the lag and its effect on the generation time. This is found in the example of *Bact. lactis aerogenes* and methylene blue, and in certain other cases, but the parallelism is not exact. The difficulty about obtaining more definite information is that precisely in the more interesting examples growth of the cells is accompanied by adaptation to the drug, so that the unmodified value of the generation time is almost impossible to determine with exactness. Furthermore, the lag is a function of the age of the inoculum, while the generation time, from its nature, is not. This again makes comparison in numerical terms difficult.

Different drugs have rather widely different effects on the total population. For varying concentrations of the same drug, however, the total population changes in parallel with the growth rate, a result which can be accounted for by a calculation based upon quite simple assumptions.†

Another important specific action of certain drugs is on the cell division probability. Where this is much reduced without a corresponding reduction in the rate of the synthetic processes, the cells may grow to great length and assume the form of filaments. In certain examples this effect may be so pronounced that the whole culture appears in the form of a few enormously long tangled threads. The whole matter is of such interest from the point of view of cell organization that it will be dealt with in a special chapter (see X).

4. Influence of drug concentration

This subject can be dealt with adequately only in relation to the detailed discussion of individual quantitative phenomena. Various examples will be considered in subsequent sections. Here, however, certain general results will be indicated.

The earlier literature is to a considerable extent concerned with the question of 'disinfection'. Many of the results are based upon experiments in which the concentration necessary to suppress visible growth in a standard test is determined. Other measurements relate to the rate at which cells die when exposed to a given concentration of the antiseptic. One of the standard ways of expressing results

† A. Poole and C. N. Hinshelwood, *J. Chem. Soc.*, 1940, 1565.

has been to set the disinfectant action proportional to c^n, n being a power of the concentration, c, of the substance under test.† This power law, however, is in need of a rational interpretation, since n often comes out to be quite considerable, 3, 4, or 5, and this can hardly have a real theoretical significance. n, moreover, is not necessarily integral: and it would therefore seem that the power law must be an approximation for a law of a rather different form.

Suppose a certain concentration c_0 of the drug can be tolerated by the cell, as a result of the operation of neutralizing mechanisms of some kind. The antibacterial action will then be proportional to $c-c_0$. Representing this action by D, we shall have

$$D = a(c-c_0),$$
$$\log D = \log a + \log(c-c_0),$$
$$d\log D/dc = 1/(c-c_0).$$

If we also choose to write

$$D = bc^n,$$

we have

$$\log D = \log b + n\log c,$$
$$d\log D/dc = n/c,$$

and to make the two methods consistent we must set

$$n/c = 1/(c-c_0),$$

or

$$n = c/(c-c_0).$$

Now suppose c_0 is considerable, then $c-c_0$ may be quite small compared with c over the range where the drug action first begins to be considerable, and n will appear large. It will not, however, appear constant, though the range of c values in which D rises from zero to practically infinity may be very small and make these variations in n difficult to observe.

Tolerance to concentrations up to a fairly well-defined threshold, followed by very steep rise in the antibacterial action, is in fact quite often observed. The different types of concentration-action curve found for *Bact. lactis aerogenes* have been summarized by the diagrams in Fig. 24.‡ The initial tolerance is shown in type 2.

As will appear more clearly later, this initial tolerance is in certain kinds of example to be expected theoretically. It will be expedient here to refer in anticipation to the results obtained with various

† See Topley and Wilson, op. cit. ‡ Poole and Hinshelwood, loc. cit.

strains of *Bact. lactis aerogenes* trained to various concentrations of proflavine. Fig. 41† shows the lag of each strain as a function of the proflavine concentration. It is evident that up to a certain

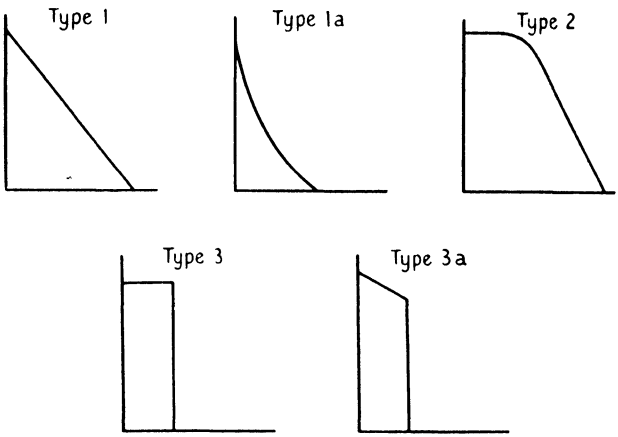


FIG. 24. Relation of growth rate (ordinates) and concentration of drug.

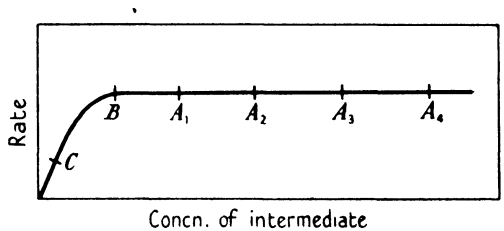


FIG. 25. Growth rate and intermediate concentration.

threshold there is little effect, after which the lag is raised rapidly towards indefinitely great values. The explanation, which we shall develop later, depends upon the idea that the proflavine interferes with the provision of the intermediate metabolite for a certain cell enzyme system. The rate of functioning of this particular system is related to the concentration of the intermediate according to a curve resembling that in Fig. 25. Normally the concentration of the intermediate in the cell has a value such as A_1, A_2,...; it can be reduced to B before the inhibition by the drug is manifest. For

† p. 139.

different strains, therefore, there will be tolerances proportional to A_1B, A_2B,...; but higher drug concentrations cause a fall to some such point as C, the observed inhibition increasing steeply as we pass from B to C. If the value for the normal cells were B, then the value of n in the power expression would be small: as we pass from B to A_1, A_2,... the value of n must rise rapidly. To quote a single numerical example: with a certain strain of cells, 100 mg./l. proflavine causes an increase in the lag in the ratio of 1·0 to 1·1; 150 mg./l., i.e. an extra 50 mg./l., then increases the lag in the ratio 1:10. This could be interpreted as indicating a value of n between 5 and 6. But such a figure would have no rational basis. The example quoted is an extreme one, since it refers to an artificially immunized strain of cells. But it is unquestionable that cells possess sometimes a degree of natural immunity to particular kinds of antibacterial agent, determined by their general species character, or more especially by their history. The mode of nutrition, which determines the enzyme balance of the cells, is probably the all-important factor. *Corynebacterium diphtheriae* strains show varying degrees of resistance to pantoyltaurine. Some strains require for their growth the addition of pantothenate to the medium: these are the most sensitive. Some can be trained to dispense with the added pantothenate: these prove to be naturally resistant to the pantoyltaurine, even though they have never been actually trained to growth in its presence.†

When there is no threshold concentration it is rather remarkable that the effect of the drug is often given by a simple linear relation, as in Fig. 24, type 1. For example, the action of alcohols on the growth rate of *Bact. lactis aerogenes* is given by

$$k = k_0 - ap,$$

where k_0 is the growth rate found in the absence of the alcohol, and k that found at concentration p, a being a constant.‡ This is illustrated in Fig. 26. If the association of the drug and the cell material is governed by an adsorption isotherm, one would expect a linear relation to hold over a certain range, but perhaps not quite so well as appears, for example, in Fig. 26. On this point two comments may, however, be made. In the first place, the relation is not always linear, but may conform rather to type 1 a of Fig. 24. In the second

† H. McIlwain, *Biochem. J.*, 1943, **37**, xiii (meeting abstract); *Brit. J. Exp. Path.*, 1943, **24**, 203, 212.

‡ S. Dagley and C. N. Hinshelwood, *J. Chem. Soc.*, 1938, 1942.

place, we must perhaps envisage the possibility that the functioning of the cell is reduced to zero, before the material upon which the

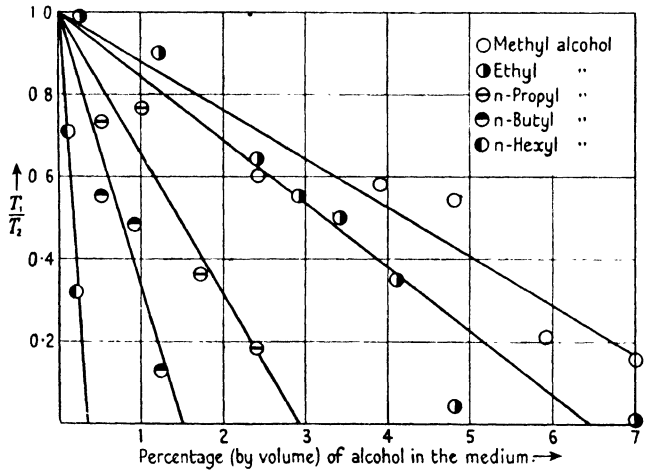


FIG. 26. Reduction in growth rate by alcohols.

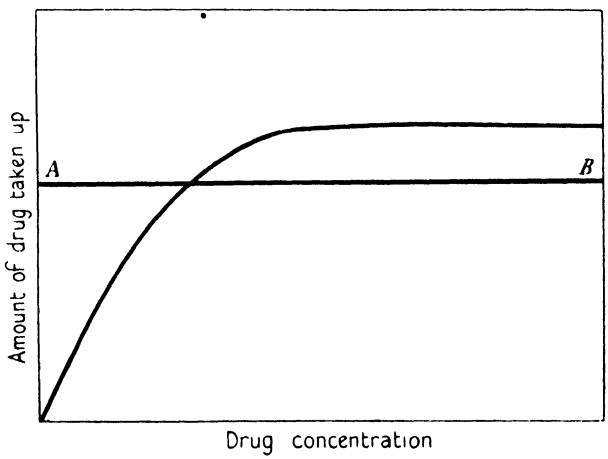


FIG. 27.

drug is taken up is saturated with adsorbed molecules. If in Fig. 27 the level AB represents that at which adsorbed drug reduces the activity of the enzyme to zero, it is evident that the linear relation for the drug action does not necessarily imply that the isotherm remains

linear up to saturation. A rather naïve interpretation of this behaviour could be suggested. If the normal substrate molecules, whose access to the enzyme substance the drug impedes, were large compared with the drug molecules themselves, the adsorption of the former could be completely prevented while there was still accommodation left for the latter.

Referring once more to Fig. 24, type 3 represents the case where the drug inhibits a lag phase reaction, so that the cell may die before growth can set in: this case was considered in the last section. Type 3a represents a combination of type 1 and type 3.

While dealing with the relation between bacterial growth and drug concentration, reference must be made to a special kind of relation encountered, for example, in the action of sulphanilamide or sulphaguanidine on *Bact. lactis aerogenes*. The growth rate falls with increasing drug concentration to a limit below which no amount of drug will reduce it. There seem to be some growth centres immune to or inaccessible to the drug. On the face of it, there are two possible explanations. We might here have the inverse of the case discussed above. If the drug molecules are larger than the normal substrate molecules, the surface of the enzyme might refuse to accept more of them, while still having spaces for the normal clientèle. On the other hand, what is more likely, at least in the present example, is that there are really two alternative mechanisms by which growth occurs, and that one is less sensitive to the sulphonamide action than the other. The whole question of sulphonamide action will be discussed more fully in another section.

The further consideration of the relation of drug action to concentration will be deferred until we deal with the subject of adaptation. It will then be possible, by more quantitative treatment, to set the whole question in a much clearer light.

5. Behaviour of homologous series of alcohols

This is an interlude to the main theme, but is introduced here because it brings us some information about the mode of interaction of the alcohol and the bacterial substance.

In Fig. 28 are plotted three sets of results for the inhibiting power of alcohols as a function of the number of carbon atoms which they contain. The results refer to the toxic effect on potato tissue,† on

† W. S. Stiles, *Introduction to Plant Physiology*, 1936, p. 81.

Bact. typhosum,† and on the growth rate of *Bact. lactis aerogenes*.‡ For the latter, the inhibiting power is measured by the value of the constant a of the formula on p. 104. It is evident that there is an accurate linear relation between the logarithm of the inhibiting power

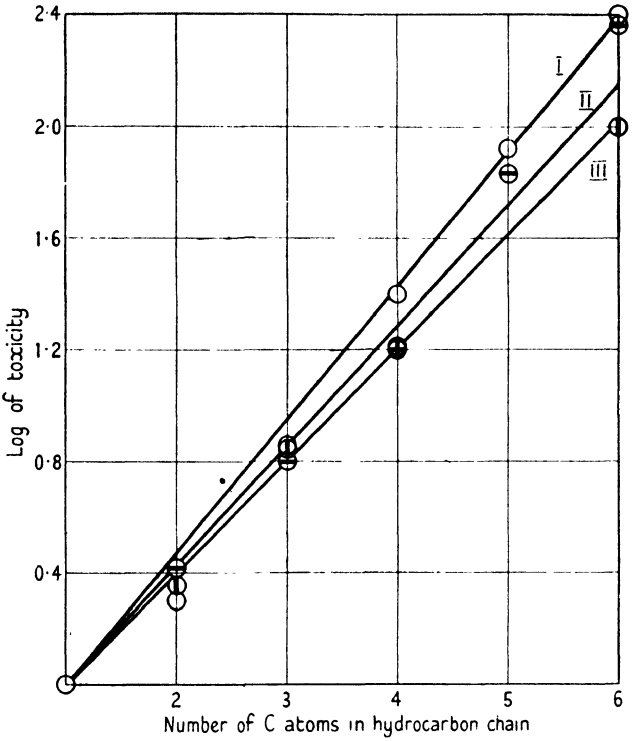


FIG. 28. Toxic action of alcohols and number of carbon atoms.

I. *Bact. typhosum*. II. Potato tissue. III. *Bact. lactis aerogenes*.

and the number of carbon atoms in the chain. This can be accounted for if we make the basic assumption that there is an interaction between each individual —CH_2 group of the alcohol and some structural element of the bacterial substance. Hydrocarbons do not dissolve in water: alcohols do. Therefore the interaction between water and an alcohol may be referred to the hydroxyl group. To remove an alcohol molecule from water will require expenditure of

† F. W. Tilley and J. M. Schaffer, *J. Bact.*, 1926, **12**, 303.
‡ S. Dagley and C. N. Hinshelwood, loc. cit.

an energy, E, approximately independent of the chain length. The number of alcohol molecules able to leave an aqueous phase in a given time will therefore be proportional to $e^{-E/RT}$. On the other hand, hydroxylic substances as such do not inhibit bacterial growth (compare carbohydrates): therefore the inhibition must be referred to the hydrocarbon chain. If each element of this is individually attached to the cell substance it will require an activation energy nQ to detach the alcohol molecule, n being the number of CH_2 groups, and Q the energy required to detach each one. If c_1 and c_2 respectively are the concentrations of alcohol in water and in the organism, we have as a condition of equilibrium:

$$A_1 c_1 e^{-E/RT} = A_2 c_2 e^{-nQ/RT},$$

whence

$$c_2/c_1 = \text{const.}\ e^{-E/RT} e^{nQ/RT}.$$

For a given value of c_1 the toxicity will depend upon c_2, and denoting it by θ we have

$$\theta \propto e^{nQ/RT},$$

or

$$\log \theta \propto n.$$

The independent attachment of each CH_2 unit means that the blocking power of the long chain compounds is very considerable. It is interesting to reflect on the growth sustaining capacity of the corresponding compounds which possess a hydroxyl group attached to most of the carbon atoms.

6. Adaptation of cells to resist the action of drugs. General Survey

One of the most remarkable phenomena in the whole range of bacteriology is the completeness, rapidity, and, in some examples, permanency with which cells may become immune to the further action of drugs after they have been grown in their presence.

The adaptive process, although very common, does not invariably operate. Thus *Bact. lactis aerogenes*, although it rapidly acquires resistance to sulphonamides, acridine derivatives, propamidine, potassium tellurite, triphenylmethane dyes, methylene blue, and other inhibitors, may be subcultured as much as 100 times in presence of a partially inhibitory concentration of phenol without showing any recovery whatever towards a normal growth rate.

Innumerable examples of acquired resistance are recorded in the

literature, and range over organisms of every type and over inorganic substances such as mercury, arsenic, and copper salts,† lysozyme of tears or sputum,‡ organic substances of most varied structure, including penicillin§ and the well-known chemotherapeutic agents.

The speed of adaptation varies, but may be very great: thus Fleming and Allison found that one single subculture was enough to give *Mycobacterium lysodeikticus* a high degree of resistance to the bacteriolytic action of tissues containing lysozyme. *Bact. lactis aerogenes* acquires moderate degrees of resistance to proflavine with great ease. It can also acquire very high degrees of resistance indeed, but these are only attained after long continued subculture in presence of the drug.

Adaptation, once acquired, may be retained tenaciously during repeated subcultures in complete absence of any drug to keep the cells in training: on the other hand, it may be lost, sometimes rapidly, sometimes slowly: sometimes as a result of specific treatment of the cells and sometimes in what appears to be a spontaneous or even unpredictable manner. Penfold,‖ speaking of acquired fermentative properties of bacteria, observed with his usual penetration that the longer a character is impressed the longer it is retained, and that the more easily the cells take on a new character, the longer they retain it. The same is generally true of drug resistance. The general pattern of adaptation and loss of adaptation becomes rather clearer in the light of quantitative measurements, and will be more fully discussed at a later stage.

Adaptation, in the true sense of the word, only occurs during actual increase in the bacterial substance. This statement also will require fuller discussion in the light of detailed experimental results: it is of considerable theoretical importance.

For the quantitative investigation of adaptive phenomena it is convenient to measure lag, mean generation time, and total bacterial population as a function of the serial number of the subculture in presence of drug at a fixed concentration. Since the lag is a function of the inoculum age, it is also useful to determine the lag-age relationship for the successive subcultures. When the cells have been trained as completely as possible at a given drug concentration, $\bar{m}$, it is

† Cf. E. K. Borman, *J. Bact.*, 1932, **23**, 315.
‡ A. Fleming and V. D. Allison, *Brit. J. Exp. Path.*, 1927, **8**, 214.
§ *Inter alia*, M. Demerec, *Proc. Nat. Acad. Sci.*, 1945, **31**, 16.
‖ W. J. Penfold, *J. Path. Bact.*, 1910, **14**, 406.

informative to test the trained cells at a whole range of drug concentrations, using for the purpose cells of such an age that the lag has, for a given value of the drug concentration, the minimum possible value. This is done for a whole series of values of $\bar{m}$ itself, and the results yield a complete family of lag-concentration curves for the various trained strains. When the adapted strains have been tested, it is usually informative to carry out experiments on reversion. The strain is subcultured serially in the absence of any of the drug, and, at appropriate intervals, is re-tested at various drug concentrations in the above ways. If spontaneous reversion does not occur, various means of inducing it may be tried, such as culture in presence of other antibacterial agents, or in media differing in composition from the one to which the cells are normally adapted.

Experiments on cross adaptation may then be tried: cells trained to one drug being tested in presence of others.

Another very important question is that of the extent to which drug-adapted cells may differ from normal cells in respects other than drug resistance. The enzymatic properties of the adapted strains can be investigated in various ways. Definite differences seem, in certain examples, to be detectable, and these have an important bearing on the theoretical interpretation of the changes which occur during the training process (see next chapter, § 13).

7. Typical examples of drug adaptation. (*a*) *Bact. lactis aerogenes* and proflavine†

In a synthetic medium of glucose, ammonium sulphate, phosphate, and magnesium sulphate the mean generation time of *Bact. lactis aerogenes* is 33 minutes. The influence of proflavine at concentrations of 43 mg./l. is initially to increase the value markedly. Successive subcultures are attended by a rapid return to the normal.

Serial subculture number	*Mean generation time (minutes)*
0	55
11	41
16	36
32	34

During the first subculture in presence of the drug long filamentous cells are often formed, which show that the drug has a specifically

† D. S. Davies, C. N. Hinshelwood, and J. M. G. Pryce, *Trans. Faraday Soc.*, 1944, **40**, 397; 1945, **41**, 163, 778; J. M. G. Pryce, D. S. Davies, and C. N. Hinshelwood, ibid., 1945, **41**, 465.

inhibitory effect on division (see Chapter X). On subculture the tendency to give abnormal forms very soon vanishes. The total bacterial population is reduced by the drug: even when the cells are completely trained in other respects, the total population remains low compared with the normal. Thus it seems that a secondary

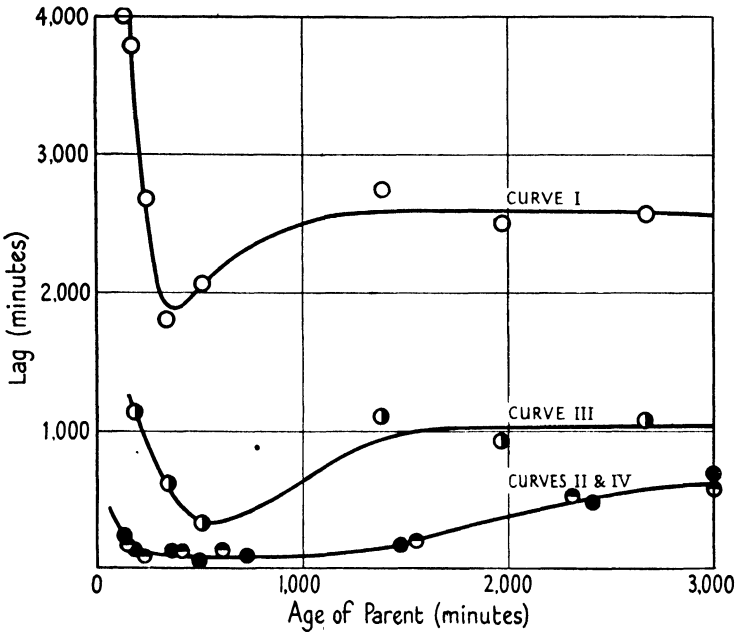


FIG. 29. Lag-age curves of *Bact. lactis aerogenes* in presence of proflavine.

I. Untrained cells: 43 mg./l. II. No drug. III. Untrained cells: 43 mg./l. proflavine and filtrate. IV. Trained cells: 43 mg./l.

action of the proflavine is to inhibit the mechanism whereby the cells usually deal with their toxic metabolic products. Immunity to this action does not appear to be acquired.

The most striking effect of the proflavine is upon the lag, which is much increased, rising steeply when the concentration exceeds about 20 mg./l. After a number of subcultures in presence of the drug the lag returns to a completely normal value. Some typical lag-age curves are shown in Fig. 29. Curve I refers to the untrained cells grown in presence of 43 mg./l. of proflavine, curve II to normal cells in absence of drug, and curve IV to the trained cells tested, after

32 passages, in 43 mg./l. proflavine. The coincidence of curves II and IV shows the completeness of the adaptation.

The lag-concentration curves for a series of adapted strains are given in Fig. 41 of the next chapter. They are seen to constitute a related family, the spacing between successive curves being equal to the difference between the corresponding concentrations of drug used in training. The discussion of this family of curves is deferred until we deal with the quantitative theory of the adaptation.

The dependence of *Bact. lactis aerogenes* upon a diffusible intermediate which accumulates in the medium has already been discussed (p. 47). The increase in lag caused by proflavine suggests that one of the results of its action is to impede the formation of this active substance. In accord with this idea we find that addition of sterilized filtrate from normal culture does indeed to some extent counteract the influence of the drug, as is shown by curve III in Fig. 29 which refers to the first subculture of untrained cells in 43 mg./l. proflavine, as for curve I, but with addition of filtrate.

A fact which is of some theoretical importance is that cells which have been trained to proflavine, even at high concentrations, show a perfectly normal lag and growth rate when returned to their normal medium.

Fig. 30 shows a typical set of results on the loss of adaptation, which is seen to be very slight.

The picture revealed by Fig. 30, however, is only part of a more complex pattern, which has required some pains to make out. As a result of a long series of experiments, first by D. S. Davies and later by J. M. G. Pryce, it has become clear. We shall have occasion later to discuss its general implications. Here the facts will be set forth without any attempt at interpretation. During the first few cell divisions in presence of moderate concentrations of proflavine, about 40 mg./l. for example, there is an extraordinarily rapid development of immunity, little or none, however, being acquired during the lag, that is to say, before actual increase of cell material begins. This is shown by the following experiment. A large inoculum of an untrained culture was transferred to a medium with 43 mg./l. of proflavine, giving a cell count in the medium which was easily measurable by direct microscopic enumeration. At the moment of inoculation, and at suitable intervals afterwards, secondary inocula were taken from this medium and transferred to new media contain-

ing also 43 mg./l. proflavine. , The lags of these secondary inocula were measured, with the results recorded below:

Time	Count of parent	Lag of inoculum transferred to	
		(a) proflavine	(b) normal medium
0	22	3,600	600
659	24	2,750	575
740	66	586	325
810	135	325	240
945	420	260	105

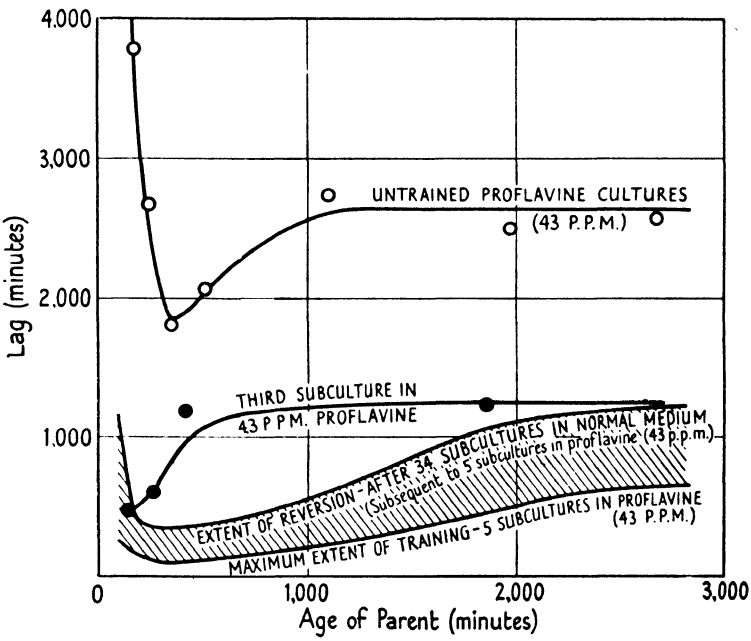


FIG. 30. Training and reversion.

At the moment when perceptible increase in numbers occurs the difference between the two last columns is still very great, showing that no serious amount of adaptation has occurred. By the time, however, the count has increased threefold, the absolute difference in the last columns has decreased from 3,000 minutes to 261 minutes, which shows that the development of the drug resistance has gone to a very considerable extent.

These cells, however, which have acquired their immunity to 43

mg./l. so rapidly and easily, are found to lose quite a considerable proportion of it if they are subcultured in the absence of proflavine. Only when the process of training has been repeated several times is the adaptation stable. At a certain stage a very limited amount of reversion is observable, and this corresponds to the state of affairs depicted in Fig. 30. With further training there is no reversion at all from adaptation to 43 mg./l. Similar behaviour is shown by

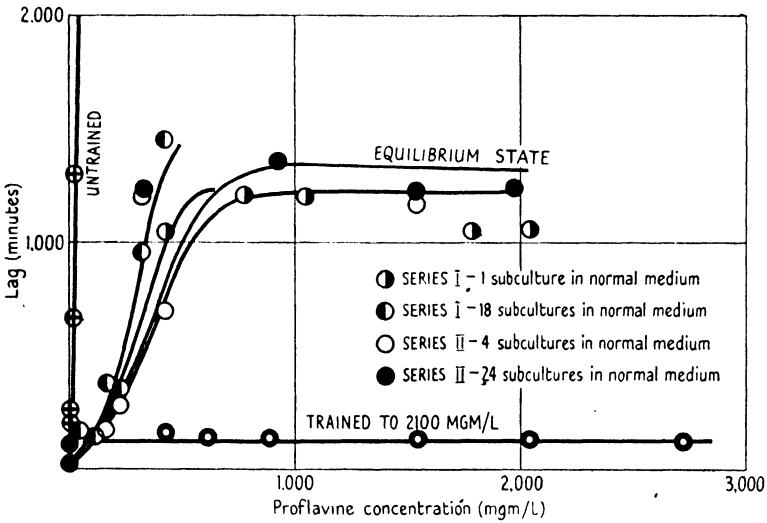


FIG. 31. Reversion of a strain adapted to 2,100 mg./l. proflavine.

strains of cells adapted to proflavine concentrations over the range represented in Fig. 31.

By transfer to progressively increasing concentrations, cells of *Bact. lactis aerogenes* can be trained to grow easily in as much as 2,000 mg./l. of proflavine, which is about 40 times the maximum concentration tolerated at all by untrained strains. The immunity, however, is attained more slowly, and is only complete after about 15 to 20 subcultures. Here again the stability of the adaptation passes through various grades. When the cells first become fully immune to the high concentration (in the sense that they show a normal lag in presence of the drug), the adaptation is not stable. On subculture in the drug-free medium, reversion occurs, not to the original untrained state, but to an equilibrium state intermediate between the untrained and the fully-trained condition. Relapse to

this equilibrium condition occurs rapidly, but beyond this no further reversion occurs even when the strain is subcultured indefinitely in the normal medium. The course of events described is illustrated by Fig. 31, which represents a fairly well-marked stage in the development of the immunity. Twenty further subcultures in the high proflavine concentration make little difference. On the other hand, a further 80 subcultures give a strain which is not only completely trained to resist 2,000 mg./l. proflavine, but is stable in the sense that it retains this immunity on passage through the drug-free medium. The lag-concentration curves of the strains which have reverted to the equilibrium state referred to above are of a different form from those characteristic of cells trained in the usual way, as may be seen by a comparison of Fig. 31 and Fig. 41.

When the 'equilibrium' strain is grown on a solid medium and single colonies are selected, subcultured, and tested for their proflavine adaptation, the fact is revealed that the 'equilibrium' population is heterogeneous. The strain consists of some cells which retain a stable immunity to quite high proflavine concentrations, and of some which have lost a considerable part of their immunity. This, of course, is not unexpected. If the cells, during training, pass from a state where reversion is possible to one where it is not, and if the transition is slow, then we must expect, in the intermediate state, a mixture of stable and unstable forms: and, in the reverted strain, a mixture of those which have lost their adaptation and those which have not. It is significant that the 'equilibrium' strain retains its character during a very large number of passages through the drug-free medium. Since it consists of a mixture of highly trained and of reverted cells, one must conclude that the latter do not increase at the expense of the former during growth in the normal medium. This being so, one cannot ascribe the loss of adaptation of the culture as a whole, during its transition from the fully-trained to the equilibrium state, to a mere shift in the balance between trained and untrained cells—which might have been assumed to occur as a result of a differential growth rate. The loss of immunity, or the retention of immunity, must therefore be a property of individual cells.

Thus we arrive at the general conclusion that training to proflavine involves, for any cell, the following stages: first, immunity incomplete; secondly, immunity complete but not stable to growth in absence of the drug; thirdly, immunity complete and stable. Even

when immunity to a concentration $\bar{m}$ is not stable, there may be stable immunity to $\bar{m}'$, a lower value. Apparently, therefore, we may have immunity complete to $\bar{m}$, but stable to $\bar{m}'$, where the latter

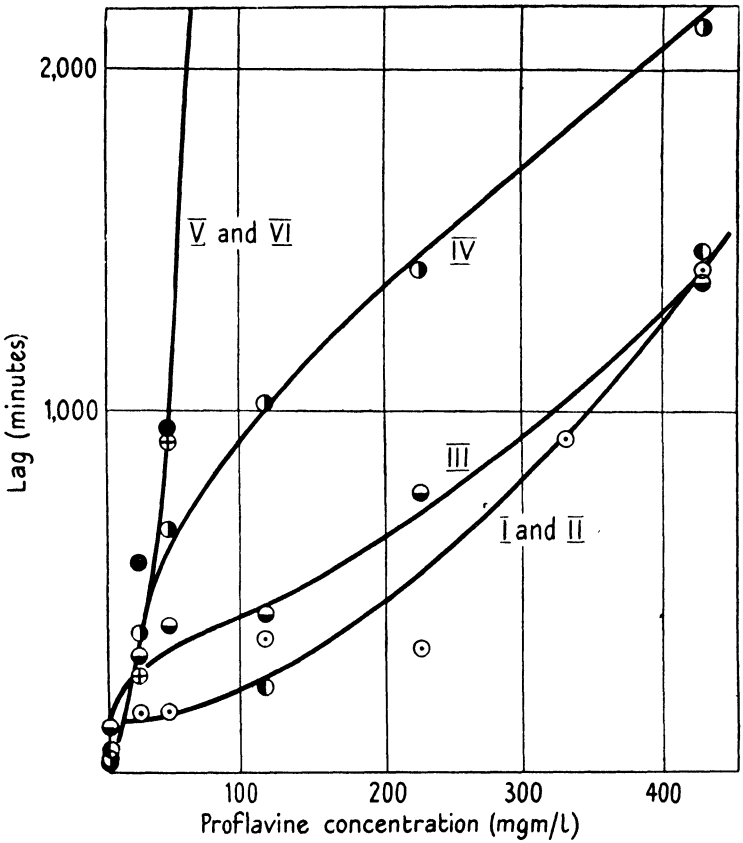


FIG. 32. Induced reversion of proflavine trained *Bact. lactis aerogenes*.
I. Trained strain. II–VI. Strains after passage through various concentrations of phenol or cresol.

number approaches more and more closely to the former the longer the training is continued. The special form of the curves in Fig. 31 can be accounted for by the heterogeneity of the 'equilibrium' reverted strain, as will be shown in another place (IX. 5).

So far, we have been speaking of spontaneous reversion of trained strains. We must now refer to the phenomenon of *induced reversion*. When proflavine-adapted strains are grown, in suitable conditions,

in presence of *m*-cresol or phenol, an induced loss of adaptation occurs. This is illustrated by the lag-concentration curves in Fig. 32, where curve I refers to the immune parent strain, and the others to strains which have been subcultured in the following concentrations of cresol or phenol: II, 0·023 per cent. cresol; III, 0·038 per cent. cresol; IV, 0·052 per cent. cresol; V, 0·07 per cent. cresol; VI, 0·14 per cent. phenol. The loss of adaptation is evident. The actual

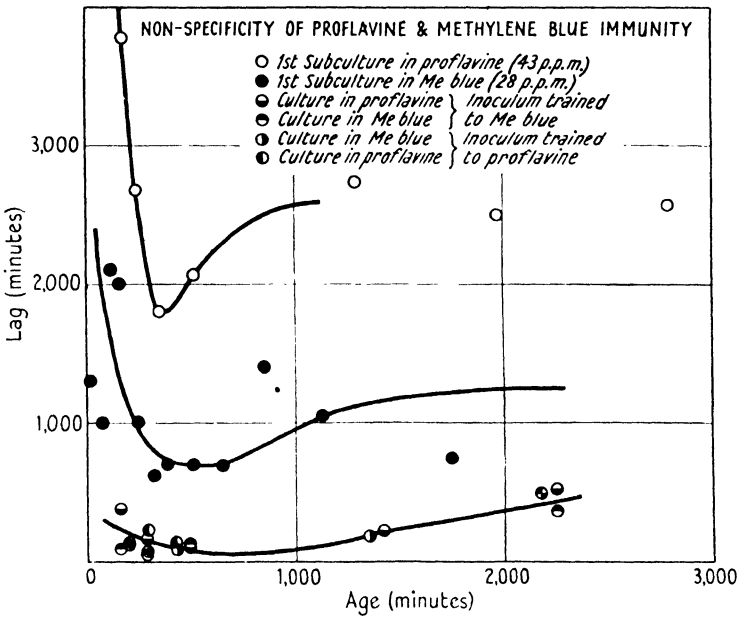


FIG. 33. Cross adaptation to proflavine and methylene blue.

form of the individual curves is explicable in terms of the hypothesis that the degree of reversion caused by one passage through the medium containing the phenolic compound is not the same for all the cells in the culture. This will be discussed more fully at a later stage (p. 219).

A certain amount of reversion is sometimes induced by culture in presence of tertiary butyl alcohol, but the degree is somewhat erratic and uncertain.

Adaptation to proflavine is associated with 'cross adaptation' to methylene blue, to other acridine derivatives, and to propamidine (partially). This subject will be more thoroughly discussed in the next chapter. For the present it will suffice to indicate the nature of the effect by reference to Fig. 33.

8. Theory of proflavine-adaptation of *Bact. lactis aerogenes*

The most obvious hypothesis to apply to any adaptive phenomenon is, at first sight, that of natural selection. This supposes cells of all possible properties, the balance of types in the total population being adjustable according as the growth of one or another is favoured. At the outset, then, we are faced with a choice between two major classes of hypothesis: one class involving assumptions about changes in the proportions of different kinds of individual in the population: the other depending upon assumptions about the way the individual cells themselves may change in response to the environment. In Chapter IV we have seen that individual enzyme balances not only can, but must, change with change in conditions of growth. Therefore, we shall begin by exploring the possibilities of the theory that individuals themselves are the primary seat of direct adaptive changes. This will form the basis of our discussion, not only of adaptation to resist drugs, but also of adaptation to new media generally. In a separate chapter we shall consider the part played by natural selection. This will be done under two heads: first, the idea that natural selection alone will account for the facts—which we shall *not* accept as a sufficient explanation: and secondly, that natural selection superposes itself on direct adaptive changes (which, of course, is inevitable if such changes occur) with results that are detectable by experiment.

The theory about adaptation to proflavine which will now be considered is based upon the idea that the drug inhibits the action of some part of the enzyme system responsible for the production of necessary growth intermediates. If the inhibition occurs at some specific stage in a sequence of processes, those members of the sequence which precede the one affected will be able to operate at their normal rate, while those which come later will be retarded, since the enzymes concerned will be starved of their normal supply of raw materials. If the formation of the intermediates required for growth is held up, the lag must be lengthened: the mean generation time will also be adversely affected. During the lag, the retarded mechanisms build up the necessary concentrations of the various substances needed to bring about division, and eventually growth starts. This does not mean, however, that the cells have become adapted: it only means that a retarded mechanism has been able to do its normal work when given increased time to do it in. Once actual

growth begins, however, the proportions of the various enzymes in the cell will change. We shall suppose that the various enzymes whose formation is retarded as a result of the action of the drug are mostly essential to the cell: and that, for this reason, division of the cell does not occur until they have been synthesized in approximately the normal standard amount. By the time this has happened

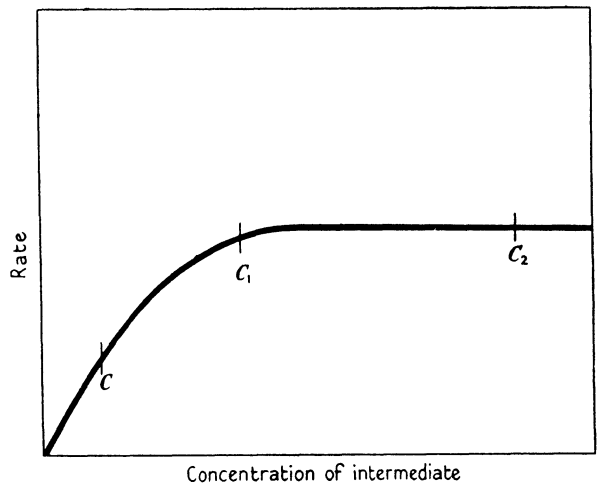


FIG. 34. Rate and intermediate concentration.

the uninhibited parts of the enzyme system will have been formed in considerably more than the normal amount per cell. When the new material synthesized is enough to outweigh the original amount, there will have been established an entirely new enzyme balance. The increased amount of one group of enzymes leads to the increased concentration in the cell of an intermediate substrate utilizable by the first enzyme of the set whose growth is inhibited. Quantitative calculations, which are developed in the next chapter, show that the enzyme balance can change until this increased concentration restores the growth rate of the inhibited set to normal. The cells are now fully adapted to resist the action of the drug. The changes in the concentration of the key intermediate are shown in Fig. 34. Before the drug acts it is c_1; while the drug acts and the cells are untrained it is c; after adaptation it returns to c_1, so long as the drug continues to act. If the cells are transferred to a medium containing

no drug, the increased amounts of certain of the enzymes cause the intermediate concentration to rise to c_2.

From the experimental fact that the trained cells show no greater activity in the normal medium than the original untrained cells, we must infer that c_1 already corresponds to nearly the limiting rate, and that further increase to c_2 does not cause any corresponding increase in the rate of operation of the enzyme system. This is not surprising in cells which have already been evolved to grow with the maximum possible efficiency in the absence of drug.†

On this basis the non-reversion of the trained strains can be partially accounted for. The increased production from the expanded part of the enzyme system does not stimulate an increase in the specific synthesis rate of the unexpanded part. The new enzyme balance established in presence of the drug is therefore not necessarily disturbed. This means that the immunity is retained. If c_1 were below the horizontal part of the curve, the increase to c_2 would cause a relative expansion of the enzymes which had been inhibited by the drug, and there would be a return towards the original proportions. According to this point of view, the retention of immunity is only possible because there is no active reason for a restoration of the original enzyme balance. If the cells are subjected to the action of some other inhibiting agent, acting upon a different set of enzymes, this sort of neutral equilibrium may well be actively disturbed and reversion may occur. The induced reversion caused by growth in phenol or cresol might well be regarded as an example of the working of this kind of process.

According to this picture, adaptation should occur with considerable rapidity, being almost complete as soon as the cell material has been well renovated. This, in considerable measure, is true. Our hypothesis, then, predicts speed, completeness, and stability of adaptation to the appropriate antibacterial agent. The observed phenomena do indeed exhibit these characters on occasion, but they exhibit others as well, which show that the picture we have sketched is only a first approximation—assuming it to be true at all. As was explained above, adaptation may be complete without being stable, and stability only seems to be acquired when the training has been continued for a much longer time than is required for mere renovation of cell material. The discussion of this matter is part of a larger

† See p. 84.

question which will be considered in another place. Here we will simply mention one possible hypothesis which seems to have several arguments in its favour.

The changed enzyme balance envisaged by the simple view outlined above must have certain secondary consequences. In the first place, all parts of the cell material form, probably, one structural unity. Expansion of one part can hardly occur without causing some reaction, possibly actual mechanical strain, upon others. Secondly, a changed enzyme balance directed solely to the correction of a disturbance caused by a drug must have certain secondary effects on the supply and demand relations of other cell functions. One can therefore imagine a cell, adapted to resist the drug, still incompletely adapted in respect of the various secondary consequences. One can well imagine that the series of secondary adjustments will require a much longer and subtler process of training than the primary enzyme expansion: and that only when they are completed will the adaptation be stable.

This extra complication is not taken into account in the simple hypothesis outlined above. The simple view nevertheless accounts for some of the principal observed results, and will be adopted for the time being as our working hypothesis. It will be developed farther in the next chapter. The question of reversion and of secondary adjustments will receive further consideration when adaptation to drugs and adaptation to utilize new sources of carbon or nitrogen are discussed from a common point of view.

Another matter of importance which should be referred to here, but which is best dealt with for the different examples under a single heading, is the fact that cells trained in various ways, including adaptation to resist proflavine, have in certain respects a demonstrably different enzyme balance—and not only in the one direct respect which is obviously related to the mode of training. To quote only a single example by way of illustration: cells trained to proflavine have a considerably reduced catalase activity (see p. 159).

9. Typical examples of drug adaptation. (*b*) *Bact. lactis aerogenes* and sulphonamides†

The class of the sulphonamides forms one of the best-known groups

† For sulphonamide inhibition generally see *inter alia*: J. Tréfouel, Mme J. Tréfouel, F. Nitti, and D. Bovet, *Compt. Rend. Soc. Biol.*, 1936, **120**, 756; J. Tréfouel, Mme J.

of antibacterial agents. The action of sulphanilamide or of sulphaguanidine on the growth of *Bact. lactis aerogenes* reveals a number of relations which must possess general significance. The behaviour

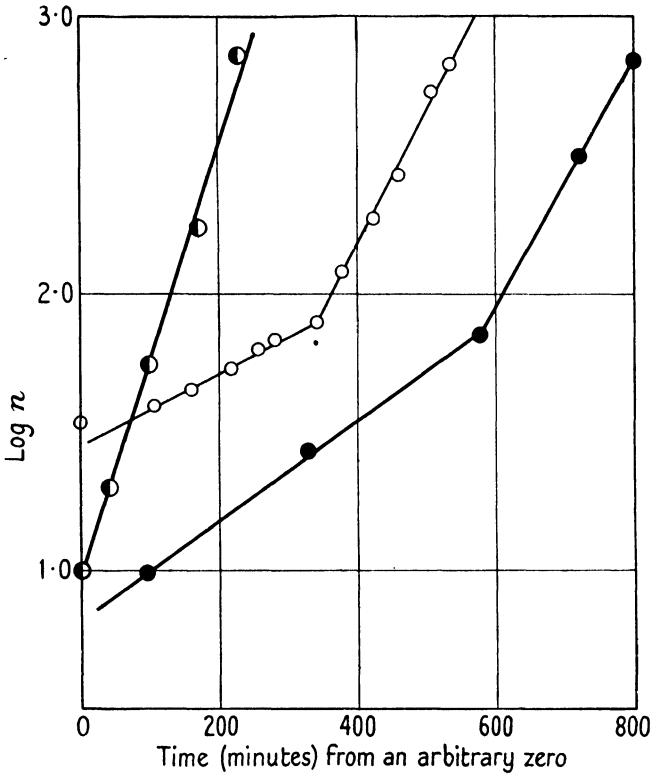


FIG. 35. Growth in presence of sulphonamide (showing characteristic composite form of growth curve).

shows in certain respects rather important contrasts with that described for the same organism and proflavine.†

Sulphanilamide lengthens the lag, and decreases the growth rate: it has little adverse effect on the total population, which may in certain circumstances even be increased by the action of the drug.

Tréfouel, F. Nitti, D. Bovet, and E. Fourneau, ibid., 1937, **122**, 258; D. D. Woods, loc. cit.; W. M. M. Kirby and L. A. Rantz, *J. Exp. Med.*, 1943, **77**, 29; E. R. Main, L. E. Shinn, and R. R. Mellon, *Proc. Soc. Exp. Biol. Med.*, 1938, **39**, 272, 591; 1939, **42**, 115; 1940, **43**, 593.

† D. S. Davies and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1943, **39**, 431.

In some respects the most interesting effect is on the form of the growth curves which, for the first subculture in presence of the sulphonamide, assume the composite form illustrated in Fig. 35. A mean generation time can be read off from each section of the curve: and the values corresponding respectively to the earlier and later stages of growth may be referred to as m.g.t. I and m.g.t. II.

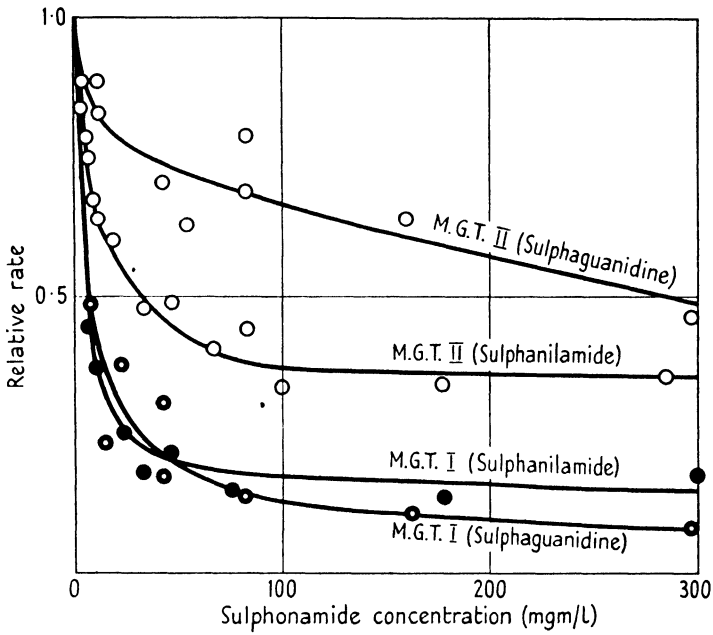


FIG. 36. Growth rate and sulphonamide concentration.

The point of intersection of the two parts of the curve will be termed the transition point.

For untrained cells both m.g.t. I and m.g.t. II increase (that is, the growth rate falls) with increasing drug concentration, but not indefinitely. For both, a well-defined limit is reached, as shown in Fig. 36, which gives the results obtained with sulphanilamide (SA) and with sulphaguanidine (SG). The ordinates represent the ratio of the normal mean generation time to that measured in presence of the drug: that is, the relative growth rate.

When the cells are subcultured serially in presence of the drug, adaptation occurs, and takes the following course. The slopes of the

two sections of the growth curve increase, but, more important, the transition point occurs earlier and earlier, soon passing below the range of easy measurement. The whole curve then assumes the normal form. In the earlier stages of adaptation, the immunity is incomplete (even although the abnormal shape of the growth curve is no longer shown): the resistance is specific to the particular sulphonamide to which the training is occurring, and it is readily lost

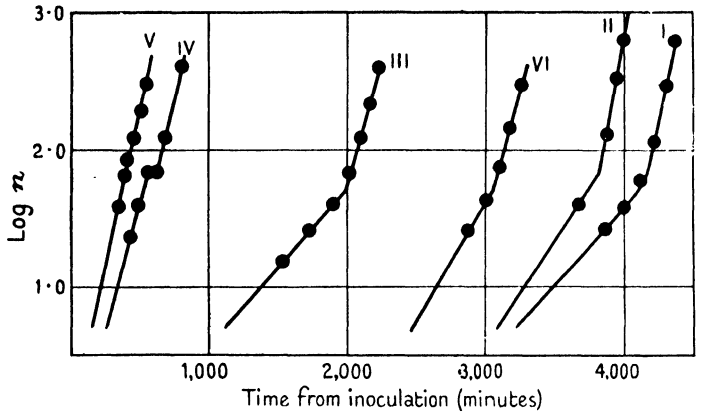


FIG. 37. Early stages of adaptation to sulphonamides.

I. Untrained growth in SG (160 p.p.m.). II. Untrained growth in SA (218 p.p.m.). III. Culture in 160 p.p.m. SG. IV. Culture in 225 p.p.m. SA; III and IV both inoculated at same time from a parent culture in 225 p.p.m. SA. V. Culture in 160 p.p.m. SG. VI. Culture in 225 p.p.m. SA; V and VI both inoculated at same time from parent culture in 160 p.p.m. SG.

on subculture in a sulphonamide-free medium (Figs. 37 and 38). After about 30 subcultures, however, the growth rate returns to the value observed in the absence of the drug; the immunity is non-specific, training to sulphanilamide giving resistance to sulphaguanidine and vice versa; and it is stable to many subcultures in the drug-free medium.

Loss of immunity may, however, be induced artificially in the stably adapted strain by culture in presence of proflavine.† This induced reversion only occurs in cells which have not had any previous opportunity of adaptation to proflavine. If they are first adapted to proflavine and then to sulphonamide, the adaptation to both may be stably retained,‡ and that to sulphonamide will survive

† D. S. Davies, C. N. Hinshelwood, and J. M. G. Pryce, loc. cit.
‡ A. M. James and C. N. Hinshelwood, in the press.

subculture at even higher concentrations of proflavine than that originally employed.

The antagonism of sulphonamides and *p*-aminobenzoic acid† has already been mentioned. The addition of the latter substance to the medium restores the growth rate in presence of sulphanilamide completely to normal.

The study of the lag relationships is complicated by a specialized

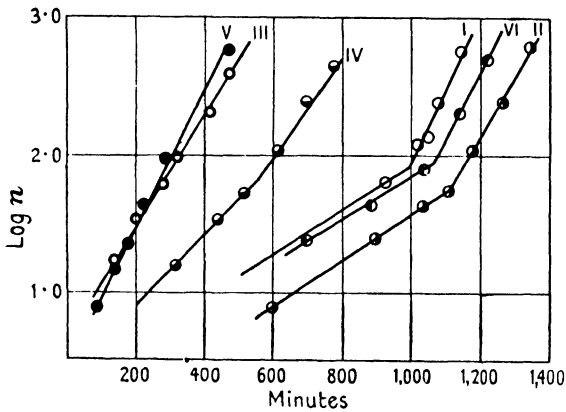


FIG. 38. Development and decay of adaptation in early stages. All cultures in 218 p.p.m. SA.

I. First culture (time zero 2,000 minutes from origin). II. Second culture in SA inoculated at transition point. III. Second subculture, inoculated at onset of stationary phase. IV. Second subculture, inoculated 800 minutes after onset of stationary phase. V. Strain made immune by three passages through SA, inoculated at onset of stationary phase. VI. Strain, from V as parent, whose adaptation has been lost after passage through normal medium.

effect: namely, that when cells of a parent culture which would show a short lag only in the normal medium are transferred to the medium containing sulphonamide, there is a short immediate burst of growth, which then slows up, *as though* the drug had required a finite time to begin to exert its action.‡ Then there is a fresh lag period, after which the definite growth phase begins. For quantitative measurements it is simpler to work with cells showing a definite lag in the drug free medium, and to study the *increase* in lag over this standard value as a function of the drug concentration.

The lag-concentration curves form a family rather different from that given by *Bact. lactis aerogenes* and proflavine. Training to a fairly

† D. D. Woods, *Brit. J. Exp. Path.*, 1940, **21**, 74.

‡ Main, *et al.*, loc. cit., but see p. 97.

low concentration of sulphanilamide gives a considerable degree of immunity to much higher concentrations. The curves show, over a considerable range, quite a large measure of independence of the drug concentration, $\bar{m}$, at which the training process is carried out. This is illustrated in Fig. 39, where it is seen that cells trained at 56, 110, and 213 mg./l. behave almost identically.

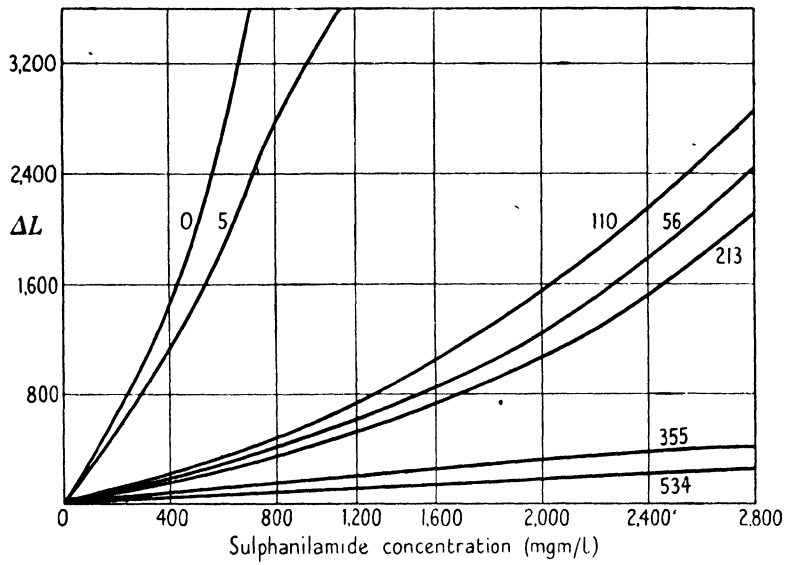


FIG. 39. *Bact. lactis aerogenes* and sulphonamide: family of lag-concentration curves.

10. Interpretation of results on sulphonamide-adaptation

The progress of the adaptation through the stages of incompleteness and instability to completeness and stability corresponds to what is found with proflavine, and, in itself, requires no further comment at the present juncture.

But the story is rather more complex than just this. In the earlier stages the adaptation is specific: cells trained to sulphaguanidine are not trained to sulphanilamide, nor vice versa. Thus the first response of the cell seems to be to develop a mechanism which is qualitatively different from that which is finally brought into play. In the final state, one may well assume that there has been an expansion of those enzymes necessary to manufacture an effective sulphonamide antagonist. This idea is consistent with the fact that the immunity is then

non-specific and complete. At the earlier stage, an alternative mode of growth seems to be used by the cell. As is discussed more fully in Chapter VII, bacteria can probably achieve their synthetic processes by many alternative routes. If one of these is blocked at a certain point, the obstruction can be by-passed, though the by-passing may depend upon the bringing into action of enzymes not normally in use. The form of the growth curves found for the first subcultures in media containing sulphonamides are very suggestive of such a situation. The matter may be made clearer by reference to Fig. 40. There are two alternative growth processes, one, the slower, possess-

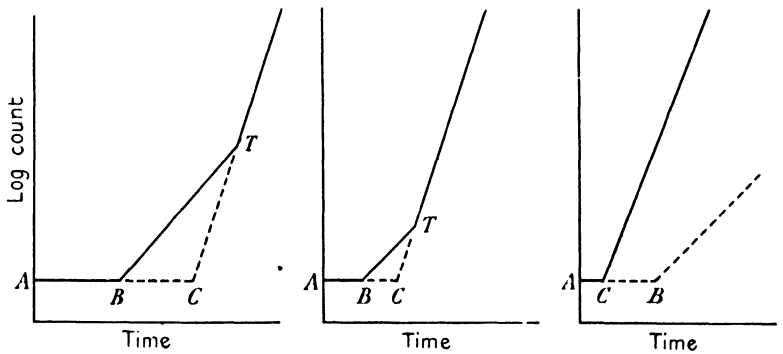


FIG. 40. Training of alternative mechanism.

ing a lag AB: the other, the faster, possessing a lag AC. Now suppose these could operate side by side and independently. (How this could happen is a matter for further consideration.)† The observed growth curve would follow the continuous line and, in the case where the faster growth mode had the longer lag, there would be a transition point as shown in the first two of the three small diagrams.

We could account formally for the behaviour during the initial stages of sulphonamide adaptation if we suppose that there exists an alternative to the normal mode of growth and that this alternative is considerably less inhibited by sulphonamides than the normal one. Initially, however, this new mode is subject to a long lag, since the necessary intermediates are not present. Ordinarily it would never have a chance to operate and develop, because growth by its more efficient competitor would be complete before it had a chance. But

† See p. 181.

the presence of the drug reverses the relative efficiencies, and the new one supersedes the old at the point T. According to this, AB is the lag, in presence of the sulphonamide of the normal growth mechanism, and AC that of the alternative. The slope of the line BT is the rate of the normal mechanism inhibited by sulphonamide, and the slope of the line CT that of the relatively insensitive alternative mechanism (CT is steeper than BT but less steep than the normal growth rate in absence of drug). As training proceeds, the lag of the alternative mechanism becomes shorter relatively to that of the normal (inhibited) one. T therefore moves downward, and finally disappears, when AC just becomes less than AB.

The sequence of curves shown by the continuous lines in the three diagrams corresponds to a well-established pattern of bacterial training, not only to sulphonamides, but also to certain changes of medium (see Chapter VII). The idea of the alternative mechanisms seems, therefore, to be of rather general applicability. It must be pointed out, however, that Fig. 40 represents an over-simplification, since the two modes of growth could hardly be treated as quite unconnected, and in practice the operation of one could hardly fail to influence the lag of the other. Indeed, the idea of two independent lags is itself not without difficulty. Nevertheless the phenomenon of broken growth curves and transition points seems to demand some such interpretation.†

What has been said is far from giving a complete account of the sulphonamide adaptation. We do not know, for example, whether the final complete immunity represents simply the perfection of the alternative growth mechanism we have been considering, or whether it depends upon a different one. There are qualitative changes which accompany the completion of the training (specificity to non-specificity) and these might suggest a completely new mechanism. But they might, on the other hand, do no more than show that the quantitative development of certain enzymes goes hand in hand with a qualitative change in their chemical texture. This question must be left open for the present. Indeed the importance of the study of sulphonamide adaptation for us at the moment lies more in the questions which it raises than in the questions which it answers.

† See also p. 180.

VI

FURTHER CONSIDERATION OF DRUG ADAPTATION

1. Hypothesis about the adaptation of cells to resist drugs

FROM the physico-chemical point of view one of the most satisfying aspects of the study of drug-adaptation is that reproducible quantitative relations can be found between lags and growth rates on the one hand and drug concentrations on the other. Each relation is characteristic of a given strain of cells, and when the bacteria are trained in presence of a given concentration, $\bar{m}$, of a drug, there are definite quantitative connexions between $\bar{m}$ and the properties of the strain produced. An attempt at an elementary mathematical formulation of these matters is clearly desirable.

The fundamental assumptions of this section are essentially the same as those of Chapter IV. Cell material is supposed to be synthesized in a series of interdependent processes, the products from each one of the sequence serving as the substrates for the next. The various parts of the cell material which increase their own substance as they do their work are referred to as enzymes, the term having thus perhaps a slightly more general meaning than is sometimes given to it.

As in the equations on pp. 76 and 85, it is postulated that the rate of accretion of substance to an enzyme is proportional to the amount of it present at any moment, and is also a function of the concentration of the intermediate derived from the previous enzyme of the sequence. This function is taken to have the general form of an adsorption isotherm, that is, the relation of rate to concentration is linear for small values of the latter and shows saturation at higher values, where rate becomes independent of concentration.

Division of the cell is assumed to occur when the increase of material reaches some critical value. As was shown in Chapter IV, when the cell reaches a steady condition, all the enzymes are built up in a constant ratio, so that any one of them could be taken as a standard and we could write *number of cells* = *constant* × *total amount of* μ*th enzyme*. In the present discussion we shall be concerned with disturbances of the enzyme balance, and we shall suppose that division has always to wait until the amount per cell of the slowest enzyme to be synthesized reaches the standard value.

This might not be true of certain inessential enzymes: but, if so, we re-define our terms by saying that we consider the sequence of the *essential* enzyme reactions of the cell.

Now we suppose that the drug interrupts the sequence by an action exerted at a stage between two particular enzymes of the series: either by interfering with the action of the $(\mu-1)$th enzyme, or with the intermediate produced by it, or by a direct action on the functioning of the μth enzyme. To simplify the notation we shall now refer to the $(\mu-1)$th enzyme as enzyme 1 and to the μth as enzyme 2. Their amounts at time t will be represented by x_1 and x_2 respectively.

Corresponding to the equations on pp. 76 and 85 we have now:

$$\frac{dx_1}{dt} = k_1 x_1, \tag{1}$$

$$n\frac{dc}{dt} = k_1' x_1 - Kcn - k_2 x_2 f(c) = 0, \tag{2}$$

$$\frac{dx_2}{dt} = k_2 x_2 f(c). \tag{3}$$

We shall suppose that division of the cell occurs when a standard amount of enzyme 2 is formed per cell, so that

$$n = \beta x_2.$$

Moreover, in the circumstances which we shall be considering, $f(c)$ may be taken to be almost linear, so that we shall replace it by c, ignoring any proportionality factor (which can be regarded as absorbed in the other constants). Equation (2) then gives

$$c = \frac{k_1' x_1}{k_2 x_2 + K\beta x_2} = \frac{x_1}{x_2}\left\{\frac{k_1'}{k_2+K\beta}\right\}. \tag{2a}$$

From (3)
$$\frac{dx_2}{dt} = \frac{k_2 k_1'}{(k_2+K\beta)} x_1;$$

but
$$x_1 = (x_1)_0 e^{k_1 t},$$

so that
$$\frac{dx_2}{dt} = \frac{k_2 k_1'}{k_2+K\beta}(x_1)_0 e^{k_1 t};$$

whence, by integration,

$$x_2 - (x_2)_0 = \frac{k_2 k_1'}{k_1(k_2+K\beta)}\{(x_1)-(x_1)_0\},$$

$(x_1)_0$ and $(x_2)_0$ being the amounts at $t = 0$. Thus

$$\frac{x_2-(x_2)_0}{x_1-(x_1)_0} = \frac{k_2 k_1'}{k_1(k_2+K\beta)} = \gamma. \tag{4}$$

The ratio x_2/x_1 tends to the limit γ, which is approached as soon as the new matter synthesized becomes great in comparison with that in the inoculum.

Now the drug will affect the value of γ, which we assume to be changed to γ'. The effect may consist in a lowering of the ratio k_1'/k_1, a lowering of k_2, or an increase in K. The first represents a reduction in the yield of active intermediate per unit amount of the enzyme 1 synthesized, i.e. a reduction in the efficiency of product formation in the general equation:

enzyme+substrate = expanded enzyme+intermediate.

The second represents a direct impairment of the functioning of enzyme 2, while the third is a waste of intermediate in reactions other than the synthesis of enzyme 2. These possibilities will be considered further in due course: for the moment all we need note is the reduction in the value of γ. From equation (4) it follows that x_2/x_1 will settle down in presence of the drug to a new ratio, and, since the amount of x_2 per cell is, on the average, kept constant by the division condition, the amount of x_1 per cell will increase.

We now consider the initial growth rate of an inoculum transferred to a medium containing drug. First, we suppose the inoculum to be taken from drug-free medium, so that

$$(x_2)_0/(x_1)_0 = \gamma.$$

But in the presence of the drug, from (4),

$$\frac{dx_2}{dx_1} = \gamma',$$

or

$$\frac{dx_2}{dt} = \gamma'\frac{dx_1}{dt};$$

therefore

$$\frac{1}{(x_2)_0}\frac{dx_2}{dt} = \frac{\gamma'}{(x_2)_0}\frac{dx_1}{dt} = \frac{\gamma'}{\gamma}\frac{1}{(x_1)_0}\frac{dx_1}{dt} = \frac{\gamma'}{\gamma}k_1.$$

Since the division of the cell keeps pace with x_2, the term $1/(x_2)_0 dx_2/dt$ represents the overall growth rate, which is seen to be reduced by the drug in the ratio γ'/γ.

Secondly, we suppose the cells to have been grown in presence of drug for some time. For the inoculum we now have

$$(x_2)_0/(x_1)_0 = \gamma', \qquad \frac{dx_2}{dt} = \gamma' \frac{dx_1}{dt},$$

$$\frac{1}{(x_2)_0}\frac{dx_2}{dt} = \frac{\gamma'}{(x_2)_0}\frac{dx_1}{dt} = \frac{\gamma'}{\gamma'}\frac{1}{(x_1)_0}\frac{dx_1}{dt} = k_1.$$

In other words, the reduction in growth rate caused by the drug has now been compensated by the change in the ratio x_2/x_1. This corresponds to a complete adaptation.

For future reference it will be convenient to note here what happens to the intermediate concentration, c, in various circumstances. From equation (2a) it is evident that if the reduction in γ is due to a lowering of k_1' or to an increase in K, then the immediate effect of the drug is to lower c. Adaptation increases x_1/x_2 and restores c to its normal value. If, on the other hand, the drug acts by lowering k_2, then c is immediately increased according to (2a) and, after adaptation, increased still more. (If K is large, the first increase is unimportant.)

The two important cases thus are: (1) the drug lowers c, and adaptation, through an increase in the ratio x_1/x_2, restores c to normal; (2) the drug lowers k_2 but the effect of this is counteracted by an increase in c which occurs on adaptation through the expansion of enzyme 1.

We now turn to the consideration of the ways in which the drug does, in fact, affect γ. If the ratio of k_1'/k_1 is reduced, it means that the drug does not impede the synthesis of the basal material of enzyme 1, but interferes with the production of the intermediate which would normally be formed. In other words, the course of the reactions occurring at enzyme 1 is deflected. This is by no means an improbability. The basal structure of the enzyme is of a protein-like character, and may be associated with prosthetic groups. One possible action of the drug might be to displace these, giving a modified protein still capable of autosynthesis but yielding different diffusible products. If P be the protein basis of the enzyme, then the action of the drug may be to replace some of the units P—X by P—Y. Then we might imagine the reaction

P—X + substrate = expanded P + intermediate utilizable by enzyme 2

to be replaced by the reaction

P—Y+substrate = expanded P+intermediate not utilizable by enzyme 2.

In this connexion one might quote the view of Albert that there is a competition between acridine drugs and hydrogen ions for negatively charged sites on protein surfaces, an idea which is introduced to account for the influence of pH on the effectiveness of the drugs of this class. (This matter is considered in more detail on p. 155.)

K would be changed if the drug combined with the active intermediate. Such an interference would be particularly likely to occur if the intermediate consisted of some molecule of very great chemical reactivity, or indeed of a free radical. Such an explanation is, however, unlikely to be a general one, since, as mentioned above, there is quite often a very marked influence of pH on the action of the drug, and this influence cannot be accounted for by a change in the molecular species present in the drug solution itself: it is due therefore to a change in the state of ionization of the protein. Thus the seat of the drug action, in such examples at least, would appear to be the protein surface itself. (Surface is here used in the rather extended sense explained on p. 10.)

The reduction of k_2 could easily be brought about by adsorption of the drug on the surface of enzyme 2, or by the formation of a drug-enzyme compound.

By which of the various means γ is changed is not relevant to the mechanism of the actual adaptive process, though of great interest to the general theory of drug action.

For the discussion of adaptive phenomena, therefore, we shall adopt an empirical expression for the drug action, which can, when convenient, be compared with any given theoretical form. We shall consider separately the two cases mentioned above, the first being where c is reduced by the initial action of the drug and restored to normal by the adaptive change: and the second where k_2 is reduced, and the rate of growth of enzyme 2 restored to normal by the increase in c resulting from the expansion of enzyme 1.

Case 1. Let the normal value of c for unadapted cells in the absence of the drug be c_1. Then we write

$$c = c_1 - \phi(m).$$

The function $\phi(m)$ of the drug concentration, m, will characterize the drug for this purpose.

After adaptation, c will have returned to c_1 even in presence of the drug. If, therefore, the cells are transferred to a medium containing no drug, there will be an immediate increase to a value given by

$$c = c_1 + \phi(m).$$

If the cells are first adapted to a concentration $\bar{m}$ and then transferred to a concentration m, the value will be given by

$$c = c_1 + \phi(\bar{m}) - \phi(m). \tag{5}$$

Case 2. Here drug and substrate compete for the surface of the enzyme. As before, let c_1 be the normal value of c for the unadapted cells in the absence of drug; in the adapted cells c will have risen to c_2, so that if $\bar{m}$ is the concentration of drug at which training occurs

$$\psi(c_1, 0) = \psi(c_2, \bar{m}), \tag{6}$$

where

$$\psi(c_1, 0) = k_2 f(c_1).$$

$\psi(c, m)$ is the function which replaces $k_2 f(c)$ when intermediate and drug are acting jointly on enzyme 2.

The total rate of functioning of enzyme 2 may be expressed

$$\text{rate} = R = R_0\{1 - \chi_1(m) + \chi_2(c)\}. \tag{7}$$

Equation (6) then becomes

$$R_0\{1 + \chi_2(c_1)\} = R_0\{1 - \chi_1(\bar{m}) + \chi_2(c_2)\}.$$

Therefore

$$\chi_2(c_2) = \chi_2(c_1) + \chi_1(\bar{m})$$

so that for trained cells transferred to another concentration m, we have

$$\text{rate} = R_0\{1 + \chi_1(\bar{m}) - \chi_1(m) + \chi_2(c_1)\}.$$

$\chi_2(c_1)$ is a constant, since c_1 is the standard value for unadapted cells in the absence of drug. We therefore write

$$R = R_0\{\text{constant} + \chi_1(\bar{m}) - \chi_1(m)\}, \tag{8}$$

which is formally similar to (5). It must be emphasized, however, that the expression (7) where the actions of drug and intermediate are represented by two independent functions can only hold over limited ranges and with express understandings about the limits of its applicability. For example, if drug has lowered R much below normal, it is reasonable to suppose that increase in c raises it again. In the absence of drug, when R was normal, a corresponding increase of c might well have less effect. (7) and (8) are therefore only taken

to apply over the range where drug and intermediate are in competition. For the subsequent discussion case 1 will be the more satisfactory to handle.

2. Influence of drugs on lag and on growth rate

The discussion of the last section dealt with the rate of growth and operation of a certain enzyme referred to for the purposes of the model as enzyme 2. It would not be any serious extension of the hypothesis to transfer the whole consideration to the growth of the entire cell: in fact the treatment adopted tells us directly, subject only to the validity of the assumptions, what will happen to the mean generation time of the cells on adaptation.

But for experimental studies of adaptive processes the mean generation time is not the most convenient quantity to handle. From its very nature, it can only be determined by experiments in which a great increase in cell material takes place: and, as we have seen, any serious increase in mass is likely to be attended by the occurrence of adaptation. Thus the measurements will always refer to cells which have already undergone some degree of adaptation.

With the lag, on the other hand, this difficulty does not arise. Up to the end of the lag phase there is very little increase in cell mass, and none at all during the major part of the time. Thus the lag is characteristic of the cell in the particular state of adaptation prevailing at the time when the transfer to the new medium is made.

The question arises, then, of the relation of the lag to the rate of enzyme action which has so far formed the basis of our calculations. In the following pages we shall make the explicit assumption that the minimum value of the lag (that is, the value which is determined by the presence of the drug alone, and is not increased by unsuitable age of the cells) is inversely proportional to the rate of an enzyme process similar to that of enzyme 2 in the model described. Actually this rate itself will probably vary during the lag period and the expression

$$\text{lag} = L = \frac{A}{\text{rate of enzyme process}} = \frac{A}{R},$$

where A is a constant, would be more correctly replaced by

$$\int_0^L R\,dt = \text{constant}.$$

We shall nevertheless use the simpler form which should serve as

a good enough approximation, since it is quite probable that R does not vary over a wide range, and in any case its average value will have properties similar to those we shall assume for the constant representative value.

The above assumption is expressly introduced as a working hypothesis, but the following general argument in its favour may be adduced.

Consider a cell in such a state that, in the absence of any drug, the lag would be minimal. Such a cell would be ready to divide, on the average, after a time equal to the mean generation time. Suppose drug to be added to the medium. There will now be a very considerable delay in the onset of normal cell division. This cannot be due to the causes which normally determine lag, that is to say to incomplete recovery of the enzymes from decay, or to the absence of the requisite intermediates—since, by hypothesis, the cells would have been ready to divide had the drug not been added. The drug-induced lag would seem, at first sight, to be nothing other than an abnormally lengthy generation time. That succeeding generation times are very considerably shorter would be attributable to the fact that, by the time they are measured, a considerable degree of adaptation has already occurred. There must be a good deal of truth in this view of the matter, but there is one difficulty about accepting it as the whole story. With drugs such as phenol there is a considerable prolongation of lag, and also a definite influence on the mean generation time. Little or no adaptive response is observable. Yet the mean generation time is much shorter than the lag itself. The latter thus appears to be something more than merely the unadapted value of the former. As we have seen in earlier discussions, intermediates are built up to a necessary threshold concentration during the lag phase: and this threshold may well have to be higher when the drug is present. The rate of building up will presumably be proportional to that of some enzyme process, so that, even here, we shall have to suppose the lag to be determined by the reciprocal of an enzyme operation rate of the kind discussed in the previous section.

3. Quantitative discussion of families of lag-concentration curves

When cells are repeatedly grown in presence of drug at a concentration $\bar{m}$ they may, as already explained, acquire a considerable degree of immunity. We imagine cells which have been thus trained

to be tested for their lag at a whole series of concentrations. For each value of $\bar{m}$ there will be a characteristic curve relating m, the test concentration, to the corresponding lag. The lag, as we have seen, depends upon the age of the inoculum: we shall consider its *minimum* value. That is to say, for all the tests we shall suppose a culture to be used of such age that in the absence of any drug the lag would be zero—or at any rate quite small. (For some purposes it is convenient to regard the minimum value of the lag not as zero but as equal to the mean generation time.) The lag-concentration curve which will be considered will be that showing ΔL as a function of the test concentration m, ΔL being the difference between the lag observed in presence of the drug and that shown by an identical inoculum in the drug-free medium.

Some typical families of curves are shown in Figs. 41, 42, 43, and 45.

In discussing the form and spacing of the curves in these families, we shall adopt as the basic hypothesis the idea that the immunity is achieved by a change in the enzyme balance in the cells. This hypothesis has already been outlined and will be taken for granted in the present section. The question whether a fundamentally different approach is possible will be considered quite separately.

We shall also adopt the assumption of the last section that the lag is expressible in the form

$$L = \frac{A}{R}, \tag{1}$$

where A is a constant (for a given type of organism and a given medium) and R is the rate of operation of an enzyme process.

R itself will depend upon the concentration, c, of the substrate which the enzyme employs, in a manner expressible approximately by a Langmuir isotherm:

$$R = \frac{kc}{1+bc}, \tag{2}$$

where k and b are constants.

The influence of the drug will be taken to change c in accordance with the discussion on p. 133:

$$c = c_1 + \phi(\bar{m}) - \phi(m), \tag{3}$$

where c_1 is the value characteristic of the unadapted cells in the absence of the drug.

Although it is an assumption that (1), (2), and (3) have these precise forms, there can be little doubt about the general shape of these expressions, so that the discussion which follows probably possesses a more general validity than the detailed arguments which have led up to it.

Substituting (3) and (2) in (1) we obtain

$$L = \frac{A}{k}\left[\frac{1}{c_1+\phi(\bar{m})-\phi(m)}+b\right].$$

If L_0 is the value of L when $m = 0$

$$\Delta L = L-L_0 = \frac{A}{k}\left[\frac{1}{c_1+\phi(\bar{m})-\phi(m)}-\frac{1}{c_1+\phi(\bar{m})}\right]. \qquad (4)$$

Equation (4) gives the family of lag-concentration curves for the series of strains which have been adapted to various drug concentrations.

4. Discussion of a typical example of a family of lag-concentration curves

One of the more thoroughly studied examples is that of *Bact. lactis aerogenes* adapted to various concentrations of proflavine (2.8 diaminoacridine).† The family of curves is shown in Fig. 41. The general form of the individual curves is in accordance with the requirement of equation (4) of the last section, the lag remaining low until m exceeds $\bar{m}$ by a certain amount, after which the lag rises steeply towards infinite values.

Furthermore the spacing of the separate curves of the family is well accounted for if $\phi(m)$ is taken to be a simple linear function of m: $\phi(m) = fm$ where f is a constant. The curves in Fig. 41 are reproduced quite reasonably well by the formula:

$$\frac{1}{(L-L_0)} = 1{\cdot}0\times 10^{-4}\{(\bar{m}+54)^2/m-(\bar{m}+54)\},$$

which is derived from equation (4) of the last section by writing $\phi(m) = fm$, and $c_1 = 54f$.

The mode of spacing of the curves can be seen from equation (4) itself. L becomes infinite when $c_1+\phi(\bar{m})-\phi(m) = 0$; that is to say, in the present example when $c_1+f\bar{m} = fm$, or when $m = 54+\bar{m}$.

† D. S. Davies, C. N. Hinshelwood, and J. M. G. Pryce, *Trans. Faraday Soc.*, 1944, **40**, 397; 1945, **41**, 163, 778; J. M. G. Pryce, D. S. Davies, and C. N. Hinshelwood, ibid., 1945, **41**, 465.

If L is large compared with L_0, without actually becoming infinite, we shall have approximately:

$$L \doteq \frac{\text{constant}}{c_1 + f\bar{m} - fm},$$

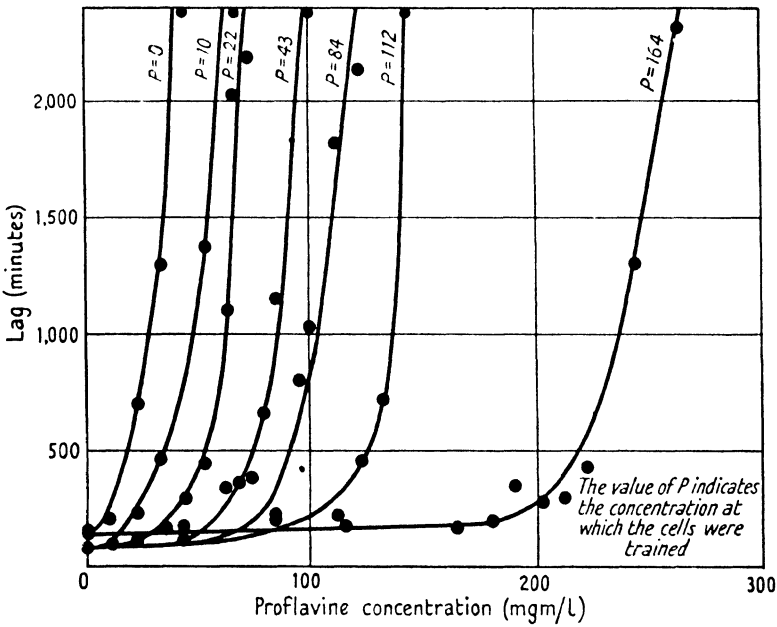


FIG. 41. Family of lag-concentration curves for trained strains of *Bact. lactis aerogenes* in proflavine.

and if m_s is the drug concentration at which the lag attains some assigned large value, for example 1,000 minutes, we shall have

$$m_s - \bar{m} = \text{constant}.$$

That this relation is rather closely satisfied is shown by the agreement of the last two columns of the following table.

m_{1000}	$m_{1000} - 40$	$\bar{m}$
30	−10	0
50	10	10
62	22	22
84	42	43
114	74	84
137	97	112
242	202	164
490	450	430

These results lend strong support to the conclusion that the adaptation always restores c to the normal level characteristic of the normal cells in absence of the drug.

The behaviour of c, the intermediate concentration, illustrated by Fig. 44 may now be considered more fully. c_1 is the normal value. The action of the drug lowers it to c_2. Adaptation restores it to a point which will be provisionally indicated by c_2'. In the drug-free medium the adapted cells develop a concentration c_1', but, from the nature of the isotherm, this enhanced value produces no corresponding increase of growth rate. The interval c_1 to c_1', while not representing any increased speed of action (decreased lag) in the ordinary medium, corresponds to a considerable degree of tolerance to the drug.

The point c_1, as previously suggested, must lie just near the shoulder of the curve, since, on the one hand, trained cells show no advantage over untrained cells when transferred to drug-free medium, and, on the other hand, untrained cells show little tolerance to the drug before the lag begins to rise. The former fact shows that c_1 cannot lie far down the sloping portion of the curve: the latter that it cannot lie far along the horizontal part.

Furthermore it now seems that the point c_2' is in fact coincident with c_1. This was tacitly assumed in the previous discussion, but is now confirmed by the experimental fact that all the curves; including that for the untrained cells, constitute a unique family, as shown by the above table.

5. Form and spacing of the curves in a family

With *Bact. lactis aerogenes* and proflavine, both the form of the individual curves and the actual spacing of the various curves are fairly satisfactorily accounted for with the help of the assumption that $\phi(m)$ is a linear function of m. This simplest type of relation is, however, not always observed. Three principal types are in fact encountered:

(1) The spacing of the curves is exactly adjusted to the value of $\bar{m}$, the concentration at which the cells are trained. Thus if m_s is the drug concentration which causes a lag of s (s being large) then $(m_s - \bar{m})$ is constant throughout the family. The example just considered, of *Bact. lactis aerogenes* and proflavine comes under this head.

(2) The curves become more and more widely separated with successive equal increments in $\bar{m}$. This is shown by the results found by J. M. G. Pryce† for *Bact. lactis aerogenes* and potassium tellurite, which are reproduced in Fig. 42. For lags of 1,000 minutes it is found that

$$m_s = 7{\cdot}1\bar{m} + 6.$$

(3) The adaptive capacity of the cells appears to be limited and

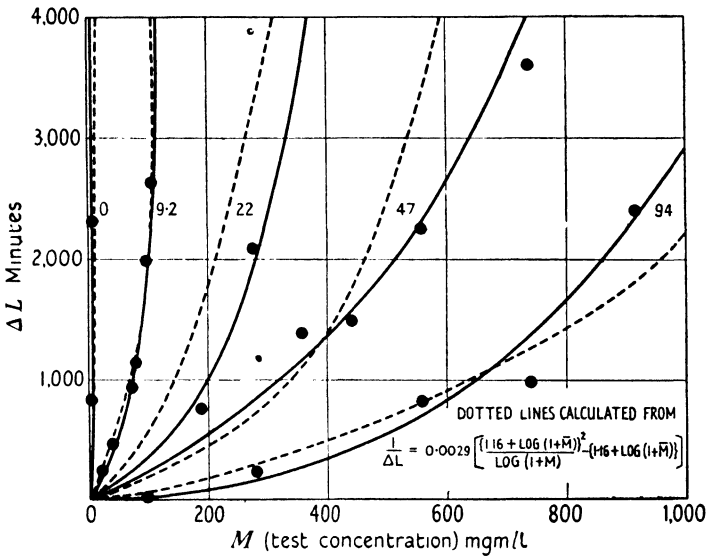


FIG. 42. Family of lag-concentration curves for trained strains of *Bact. lactis aerogenes* in potassium tellurite.

attempts to increase $\bar{m}$ become more and more difficult. This is illustrated in Fig. 43 which gives Pryce's results† for *Bact. lactis aerogenes* and propamidine. The successive curves of the family crowd together.

The family of curves obtained by D. S. Davies† for the adaptation of *Bact. lactis aerogenes* to crystal violet is shown in Fig. 45, and is of an intermediate form, beginning like type (2) and then settling down to conform to type (1).

Adaptation of the same bacterium to sulphanilamide is a rather special form of type (2), training to moderately small concentrations

† In course of publication.

conferring immunity to very much higher ones. Moreover, in certain ranges the behaviour of the trained cells seems to be nearly independent of the value of $\bar{m}$. The whole question of adaptation to sulphonamides will be separately dealt with in a later section.

Both the form and the spacing of the curves are determined by the function $\phi(m)$. Since the lag is given

$$\Delta L = \text{constant}\left[\frac{1}{c_1+\phi(\bar{m})-\phi(m)} - \frac{1}{c_1+\phi(\bar{m})}\right]$$

we may write, when ΔL is fairly large,

$$\Delta L = \frac{B}{c_1+\phi(\bar{m})-\phi(m)}.$$

If m_s is the concentration of drug which causes a lag s, then for the various strains of trained cells we have:

$$c_1+\phi(\bar{m})-\phi(m_s) = \text{constant} = E.$$

Now let $\phi(m)$ be expressed empirically as a power series

$$\phi(m) = \alpha m+\beta m^2+\gamma m^3+\ldots,$$

α, β, and γ being constants.

Then

$$\alpha m_s+\beta m_s^2+\gamma m_s^3 = E+\alpha\bar{m}+\beta\bar{m}^2+\gamma\bar{m}^3,$$

$$\alpha\frac{dm_s}{d\bar{m}}+2m_s\beta\frac{dm_s}{d\bar{m}}+3\gamma m_s^2\frac{dm_s}{d\bar{m}} = \alpha+2\beta\bar{m}+3\gamma\bar{m}^2,$$

$$\frac{dm_s}{d\bar{m}} = \frac{\alpha+2\beta\bar{m}+3\gamma\bar{m}^2}{\alpha+2\beta m_s+3\gamma m_s^2}.$$

The various types enumerated above correspond to different forms of the $\phi(m)$ function.

(1) If β and γ are zero, then $dm_s/d\bar{m} = 1$, and the spacing is uniform with that of the values of $\bar{m}$.

(2) If α is positive and the joint effect of the others negative, then, since m_s is greater than $\bar{m}$, the value of $dm_s/d\bar{m}$ is greater than unity. This means that the spacing increases as $\bar{m}$ increases.

(3) If the joint effect of the β and γ terms is positive, then $dm_s/d\bar{m}$ is less than unity and successive curves tend to crowd together with increase of $\bar{m}$.

To the extent that the $\phi(m)$ function is a purely empirical one this classification is only a formal one. But what is definitely more significant is that the same $\phi(m)$ function which describes the spacing

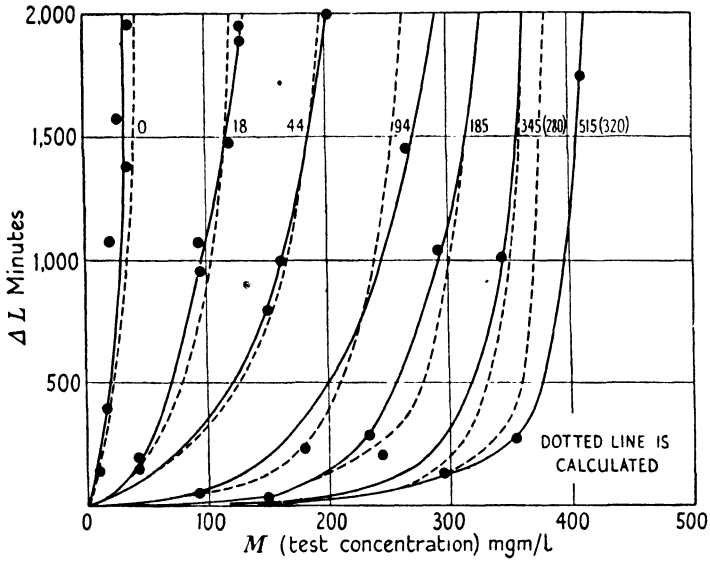


FIG. 43. Family of lag-concentration curves for trained strains of *Bact. lactis aerogenes* in propamidine.

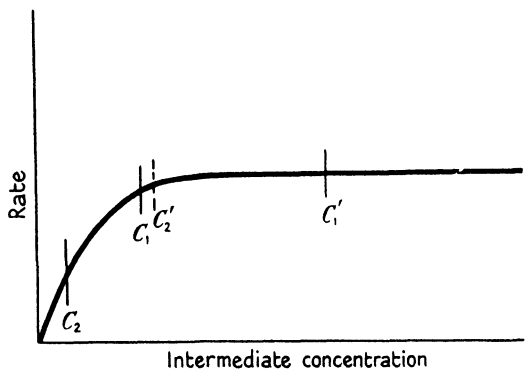


FIG. 44. Intermediate concentrations and rate.

of the curves also describes certain important properties of the individual curves themselves.

From the expression

$$\Delta L = \frac{B}{c_1 + \phi(\bar{m}) - \phi(m)},$$

we find by differentiation

$$-\frac{B}{(\Delta L)^2}\frac{\partial(\Delta L)}{\partial m} = -\phi'(m) = -(\alpha+2\beta m+3\gamma m^2),$$

$$\frac{\partial(\Delta L)}{\partial m} = \frac{(\Delta L)^2}{B}(\alpha+2\beta m+3\gamma m^2).$$

Now consider the slope of the curve of ΔL against m, measured at a standard value of ΔL. In families of type (1), the proflavine type, β and γ are zero, and therefore, for a given value of ΔL, the slope $\partial(\Delta L)/\partial m$ should be constant. That this is approximately true is shown by the numbers which follow:

$\bar{m}$ mg./l.	$\partial m/\partial(\Delta L)\times 10^3$ mg./l./min. at $\Delta L = 1000$
0	1·16
10	1·10
22	0·97
43	1·10
84	1·12
112	1·06

In families of type (2), $\alpha+2\beta m+3\gamma m^3$ decreases with increase of m. The greater $\bar{m}$, the greater the value of m required to produce a given value of ΔL, and therefore, the slope $\partial(\Delta L)/\partial m$ decreases as $\bar{m}$ increases. That successive curves of the family are of decreasing steepness is shown in Fig. 42.

For *Bact. lactis aerogenes* and potassium tellurite, Pryce found that $\phi(m)$ could be empirically represented by

$$\phi(m) = a\log(1+1{\cdot}0m).$$

This was obtained from the spacing of the curves. Since

$$\frac{B}{(\Delta L)^2}\frac{\partial(\Delta L)}{\partial m} = \phi'(m) = \frac{1{\cdot}0a}{1+1{\cdot}0m},$$

$$\frac{\partial m}{\partial(\Delta L)} = \frac{B}{(\Delta L)^2}\frac{(1+1{\cdot}0m)}{a}.$$

The inverse slope $\partial m/\partial(\Delta L)$ should increase linearly with m, the value of the drug concentration needed to produce the standard lag. The following table shows that this prediction is approximately fulfilled.

$\bar{m}$	m_{1000}	$(\partial m/\partial\Delta L)_{1000}\times 10^3$	$(3+0{\cdot}45m_{1000})$
0	4	5	5
9·2	75	30	37
22	200	93	93
47	320	230	147
94	650	295	295

A family of curves of type 3 is illustrated by the lag-concentration curves for various adapted strains of *Bact. lactis aerogenes* tested in propamidine. From the variation of m_s with $\bar{m}$ the $\phi(m)$ function can be found graphically (see next section). The curve of $\phi(m)$ against m shows an inflexion. At this point the second differential coefficient of $\phi(m)$ is zero. But since $\phi'(m)$ is proportional to $\partial(\Delta L)/\partial m$,

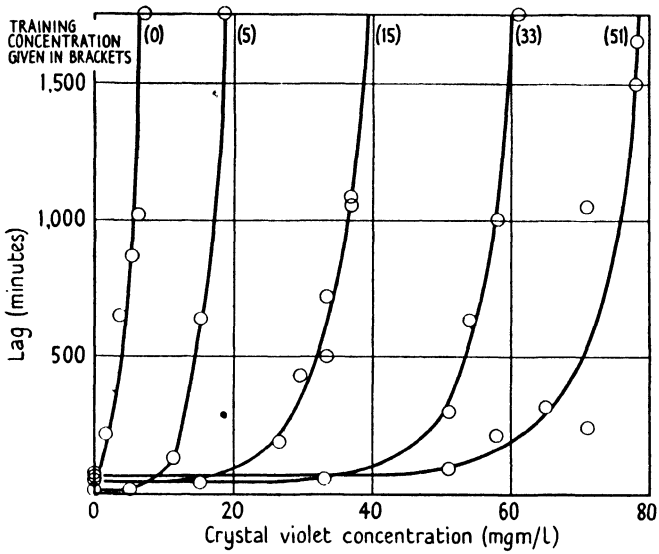


FIG. 45. Family of lag-concentration curves for trained strains of *Bact. lactis aerogenes* in crystal violet.

$\phi''(m)$ is proportional to $\partial^2\Delta L/\partial m^2$. Thus at the value of m corresponding to the point of inflexion in the $\phi(m)$, m curve the slope of the $\Delta L, m$ curve should show a maximum or minimum value—actually a minimum. For $\Delta L = 1{,}000$ this minimum occurs at about $m = 200$. The decrease and subsequent increase in the steepness of the curves at $\Delta L = 1{,}000$ can be seen in Fig. 46. The slopes are recorded in the following table derived from Pryce's experimental results.

$\bar{m}$	m_{1000}	$(\partial\Delta L/\partial m)_{1000}$
0	26	133
18	96	23
44	165	18
94	245	17
185	292	17
280	342	33
320	395	48

The position of the minimum is not very well defined, but the results are quite consistent with its being in the predicted region of $m_{1,000} = 200$.

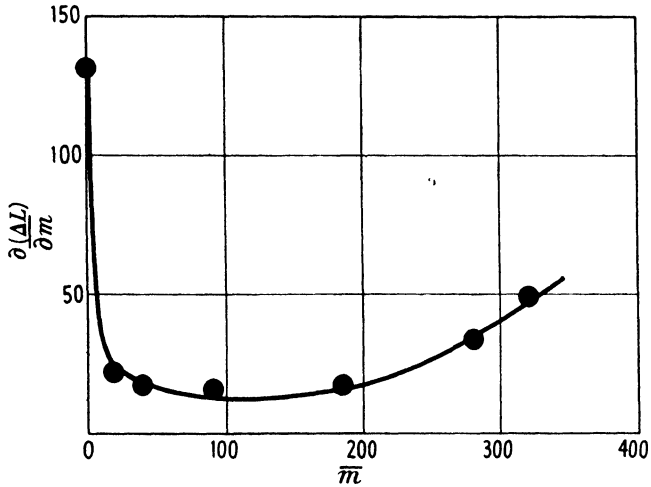


FIG. 46. Slopes of lag-concentration curves.

6. Determination of $\phi(m)$ curves

The foregoing discussion shows that the spacing of the members of a family of lag-concentration curves on the one hand, and the forms of the individual curves on the other can be satisfactorily correlated with the aid of the $\phi(m), m$ function for the system. It is convenient therefore to regard this relation as possessing at least empirical value. The methods of determining it may now be indicated.

Some standard value of ΔL is chosen and the corresponding drug concentration m_s is read off from the lag-concentration curves for each value of $\bar{m}$. If possible the relation of m_s and $\bar{m}$ is expressed by a convenient empirical equation, such as that used above for Pryce's potassium tellurite experiments:

$$m_s = 7{\cdot}1\bar{m} + 6. \tag{1}$$

For a constant value of the lag, the general equation shows that $\phi(m) - \phi(\bar{m}) = \text{constant}$. Thus

$$\phi(m_s) - \phi(\bar{m}) = \text{const.} \tag{2}$$

It is sometimes possible to find a simple form for $\phi(m)$ such that (1) follows directly from (2). This will, of course, be the form required. In the present example $\phi(m) = a\log(1+bm)$ satisfies the conditions, as may easily be verified by substitution in (2).

When an algebraical method is not convenient a graphical method may be used. If m_s is plotted against $\bar{m}$, a series of pairs of values m_{s1}, $\bar{m}_1$; m_{s2}, $\bar{m}_2$,... can be read off such that $\bar{m}_j = m_{s,j-1}$. But $\phi(m_s)-\phi(\bar{m}) = \text{constant} = C$. For the untrained cells we have $\phi(m_{s0})-\phi(0) = C$, and since $\phi(0) = 0$, we have that $\phi(m_{s0}) = C$. Then $\phi(m_{s1})-\phi(\bar{m}_1) = C$. But $\phi(\bar{m}_1)$ was chosen to be equal to $\phi(m_{s0})$. Thus $\phi(m_{s1})-\phi(m_{s0}) = C$, or $\phi(m_{s1})-C = C$, whence $\phi(m_{s1}) = 2C$. In this way the whole series of $\phi(m)$ values can be found as multiples of C. C can easily be adjusted to give the whole $\phi(m), m$ curve the required scale. From the curve so constructed the values corresponding to any particular drug concentration, m, can be read off.

7. Significance of $\phi(m)$ curves

The expression $c = c_1-\phi(m)$ was introduced because it was of convenient form to handle, and has been seen to be useful for purposes of correlation. The $\phi(m), m$ curves found in practice are of varied shape, sometimes being linear, sometimes having $\phi(m)$ increase either more or less rapidly than m, or even showing a point of inflexion, at which a more than linear increase with m succeeds a less than linear increase.

We must now inquire briefly into the possible meaning of such behaviour.

From equation (2a) of p. 130 we have

$$c = (x_1/x_2)\{k_1'/(k_2+K\beta)\},$$

and since $x_2/x_1 = \gamma$ by equation (4) of p. 131, it follows that

$$c_1 = \frac{1}{\gamma_1}\frac{k_1'}{k_2+K\beta},$$

where γ_1 is the value of γ for normal cells.

In presence of the drug k_1' will be assumed to be reduced (case 1, p. 133), so that

$$(k_1')_m = k_1'-\psi(m).$$

Therefore

$$c_1-c = \frac{1}{\gamma_1(k_2+K\beta)}\{k_1'-(k_1')_m\} = \frac{\psi(m)}{\text{constant}}.$$

Since, therefore, $$c = c_1 - \frac{\psi(m)}{\text{constant}}$$

and $$c = c_1 - \phi(m),$$

$\psi(m)$ is proportional to $\phi(m)$.

A linear course of the $\phi(m), m$ curve, therefore, corresponds to a linear reduction of k_1' with drug concentration. Such linear relations are not at all uncommon (see V. 4), and conform to known types of adsorption isotherm (see I. 5). When $\phi(m)$ increases more than linearly with m it would appear that the reduction of k_1' depends upon a kind of adsorption (of drug on the enzyme surface) where co-operative effects between the drug molecules occur (see p. 6). Less than linear proportionality suggests that adsorption of a certain amount of drug impedes the adsorption of more drug to a greater extent than it impedes the adsorption of the competing substrate. This might well occur if the drug molecules were large and the substrate molecules small. For a point of inflexion in the $\phi(m), m$ curve, the condition would be that the two effects just referred to should be present in competition with one another.

Similar considerations apply, though in a slightly more complicated way to case 2 of p. 134.

The whole subject which has been touched on in this section is worthy of much fuller study, which, however, we are not in a position to give it at present. Studies of the actual adsorption of drugs by cell material might be expected to yield results of interest.

8. Cross adaptation

Cells of *Bact. lactis aerogenes* which have become adapted to proflavine show increased resistance to inhibition by methylene blue and vice versa. Training to proflavine (2.8 diamino acridine) confers some degree of immunity to other acridines. Indeed, training to various members of the acridine group, 2.8 diamino acridine, 5 amino acridine, and 2.7 diamino acridine shows a set of reciprocal relations which are approximately quantitative.

Certain results found with *Bact. lactis aerogenes* for proflavine and methylene blue will be summarized as an illustration of the kind of relations which are characteristic of cross adaptation.†

† J. M. G. Pryce, D. S. Davies, and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1945, **41**, 465.

Methylene blue increases the lag of the cells in a synthetic medium: for untrained cells the empirical relation

$$L - L_0 = 30m \tag{1}$$

is approximately followed.

Various adapted strains give a series of lag-concentration curves which crowd together as $\bar{m}$ increases, that is to say, there is a limit to the adaptation which can occur.

Each of the trained strains, tested in proflavine, shows an immunity to this drug corresponding to what it would have acquired by direct adaptation at a concentration P'. The equivalent proflavine concentration, P', plotted against the methylene blue training concentration $\bar{m}$, gives a curve which is markedly concave to the $\bar{m}$ axis, that is to say, the equivalent proflavine immunity increases much less rapidly than $\bar{m}$. This stands in an obvious qualitative connexion with the fact that the methylene blue immunity itself tends to a limit.

It is of interest to inquire how far a quantitative relation exists. This is tested as follows.

From the equation (4) of p. 138 we have

$$L - L_0 = \frac{A}{k}\left[\frac{1}{c_1+\phi(\bar{m})-\phi(m)} - \frac{1}{c_1+\phi(\bar{m})}\right]. \tag{2}$$

The constants A/k and c_1 are obtained by experiments *with proflavine alone*. Then by comparison of equation (1) above, with (2) for $\bar{m} = 0$, a series of values of $\phi(m)$ may be found and plotted against m. The resulting curve is concave to the m axis. We now have the constants of equation (2) and the $\phi(m), m$ relation from experiments in which no methylene blue-adapted strains of the organism have been used at all. From the results, however, we are in a position to predict the behaviour of the methylene blue-trained strains both in methylene blue and in proflavine. The basic assumption of this procedure is that the modes of attack of the two drugs on the cells are identical and that the adaptive response is of one kind only for both.

The results of the attempted prediction are as follows:

(1) Cells trained at the highest possible concentration of methylene blue are calculated to show an equivalent proflavine immunity which should never exceed that corresponding to training at 54 mg./l., i.e. P' should have a limiting value of 54. Experimentally the limiting value of P' was found to be 30.

(2) The calculated lag-concentration curves for the methylene blue-trained strains crowd together in the correct manner, but the shape of the individual curves is not very well reproduced. The comparison of the calculated and the observed results is shown in Fig. 47.

The conclusion of Pryce, Davies, and Hinshelwood was: 'Summarizing the results of the calculations, we may say that they reveal

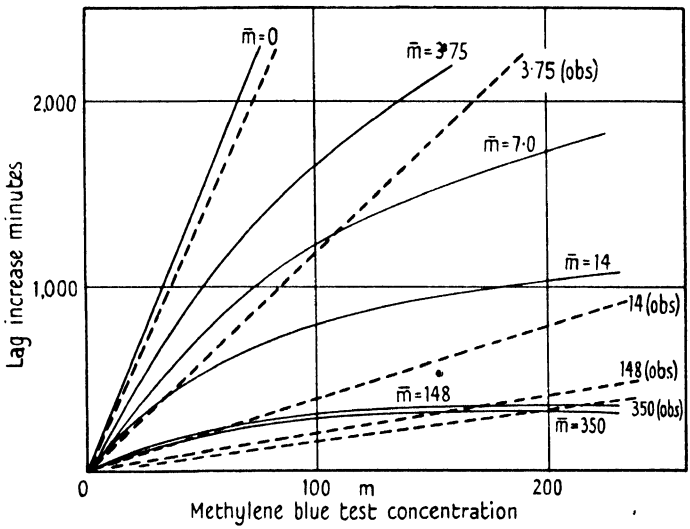


FIG. 47. Family of lag-concentration curves for trained strains in methylene blue.

a significant correspondence between the behaviour of proflavine and that of methylene blue, and allow the semi-quantitative prediction of one type of behaviour from the other. Whether better numerical agreement would be obtained with more accurate results, or whether, superposed on the common actions of the two drugs, there are specific actions to which the theory is inapplicable is hard to say at present. But the above theoretical discussion would appear to cover a not inconsiderable part of the truth.'

The whole matter, one may hope, may be clarified by future experiments.

9. Development of alternative mechanisms

In the example of adaptation of *Bact. lactis aerogenes* to sulphonamides we have seen that the development of an alternative growth

mechanism is at least a possibility. In this section a hypothetical model of such a process will be considered.†

Suppose we have the following relation of enzymes:

$$\begin{array}{l} \qquad\qquad\quad \mathrm{M_1} \\ \mathrm{I} \rightarrow c \rightarrow \mathrm{II} \rightarrow \mathrm{M_2} \searrow \\ \qquad\qquad\qquad\quad c' \quad \mathrm{III} \\ \mathrm{IV} \rightarrow \mathrm{M_2} \nearrow \end{array}$$

Enzyme I produces the intermediate $\mathrm{M_1}$ which attains in the cell the steady concentration c and which is the substrate for enzyme II. Enzymes II and IV are alternative sources of $\mathrm{M_2}$ which is utilized by enzyme III. The steady concentration of $\mathrm{M_2}$ is c', to which, normally, II makes a much greater contribution than IV. $\mathrm{M_1}$ and $\mathrm{M_2}$ stand in the relation:

$$\mathrm{M_1}+\text{enzyme II} = \text{extended enzyme II}+\mathrm{M_2},$$

so that if c drops, c' will drop in consequence.

Let the drug interfere with the production of $\mathrm{M_1}$, thereby cutting down the supply of $\mathrm{M_2}$ from II and making III relatively more dependent on IV. We assume III to be an important constituent of the cell, so that division has to await the formation of the standard amount of it.

In a medium where a steady state has been reached I and IV maintain a constant ratio. If their amounts are x_1 and x_4 respectively, then

$$x_1 = (x_1)_0 e^{Kt} \quad \text{and} \quad x_4 = (x_4)_0 e^{Kt},$$

K being the overall growth constant.

$$x_4/x_1 = \gamma. \tag{1}$$

The concentrations c and c' are determined by the factors which have been discussed before; but to simplify the calculation we are now going to neglect the loss by diffusion from the region of the enzymes. The essential result will not be changed by this.

$$dc/dt = k_1' x_1 - k_2 x_2 c = 0, \tag{2}$$

$$dc'/dt = k_2 x_2 c + k_4' x_4 - k_3 x_3 c' = 0,$$

or

$$dc'/dt = k_1' x_1 + k_4' x_4 - k_3 x_3 c' = 0. \tag{3}$$

(2) and (3) assume the synthesis and functioning of II to be com-

† D. S. Davies, A. M. James, and C. N. Hinshelwood, *Trans. Faraday Soc.*, in the press.

pletely linked. To avoid the introduction of extra symbols, the formation of $\mathbf{M}_2$ is equated to the formation of x_2

$$dx_3/dt = k_3 x_3 c' = k'_1 x_1 + k'_4 x_4 \qquad \text{from (3)}$$

and since $dx_1/dt = Kx_1$, we have

$$dx_3/dx_1 = (k'_1 x_1 + k'_4 x_4)/Kx_1 = k'_1/K + k'_4 \gamma/K. \tag{4}$$

For a culture growing under constant conditions x_3/x_1 must settle down to the same value as dx_3/dx_1, and thus for an inoculum taken from such a culture trained to a drug concentration $\bar{m}$,

$$(x_3)_0/(x_1)_0 = (k'_{1(\bar{m})} + k'_4 \gamma)/K = A_{(\bar{m})}. \tag{5}$$

If this inoculum is transferred to another drug concentration, m, it will grow in such a way that x_3/x_1 tends to a new value A_m. But *initially* the rate of growth of x_3 will be given by

$$(dx_3/dt)_0 = k'_{1(m)}(x_1)_0 + k'_4 \gamma (x_1)_0 = K_m (x_3)_0.$$

Thus
$$K_m = k'_{1(m)} \left\{\frac{(x_1)_0}{(x_3)_0}\right\}_{(\bar{m})} + k'_4 \gamma \left\{\frac{(x_1)_0}{(x_3)_0}\right\}_{(\bar{m})},$$

or
$$K_m = \left\{\frac{(x_1)_0}{(x_3)_0}\right\}_{\text{(training)}} \{k'_{1(\text{test})} + k'_4 \gamma\}. \tag{6}$$

the subscripts '*training*' and '*test*' being more explicit ways of writing $\bar{m}$ and m respectively.

k'_1 is the quantity assumed to be affected by the drug. When it is reduced there is a depression in growth rate according to (6). According to (5), however, $(x_3)_0/(x_1)_0$ will adjust itself to a new value, so that eventually

$$\{(x_3)_0/(x_1)_0\}_{(\bar{m})} \quad \text{becomes} \quad (k'_{1(m)} + k'_4 \gamma)/K$$

since the cells are now trained at the test concentration.

$K_{(m)}$ now equals K once more and complete adaptation has occurred.

So far, the present case differs little from that considered earlier, but when we come to consider the behaviour of the cells in still higher concentrations of the drug the difference appears. The smaller the factor $(k'_1 + k'_4 \gamma)$, the greater is the relative importance of $k'_4 \gamma$, since it is k'_1 which is sensitive to the drug. (5) and (6) may be written in the form

$$\frac{K_m}{K} = \frac{k'_{1(\text{test})} + k'_4 \gamma}{k'_{1(\text{training})} + k'_4 \gamma} = \frac{F(m) + B}{F(\bar{m}) + B}, \tag{7}$$

where F represents a function of the drug concentration and B is independent of the drug. As soon as $F(m)$ is small compared with B, the further reduction in $F(m)/F(\bar{m})$ is of no consequence. This means that there is a value of the training concentration $\bar{m}$ which will give complete immunity even to vastly greater values of m.†

As we have seen, strains of *Bact. lactis aerogenes* trained to sulphonamide tend to behave in a manner which, over a certain range at least, is more or less independent of the training concentration. Davies, James, and Hinshelwood,‡ with the help of some tentative approximations, found the behaviour to be in general accord with the requirements of equation (7), but did not reach a definite conclusion whether the relations could not also be accounted for by a suitable $\phi(m), m$ curve. There is, however, also the evidence of the composite growth curves in favour of some sort of alternative mechanism theory for the sulphonamide training. These, unfortunately, require an alternative mechanism theory of a rather different form, which will be best considered in relation to adaptation to change of food material.

10. Further remarks on the reversion of trained cells

In Fig. 44, if the normal concentration of an important intermediate corresponds to the point c_1, and if it is reduced by the drug, then adaptation occurs and c returns to c_1 in presence of the drug. When the cells are transferred to a drug-free medium c rises to a value corresponding to the level part of the concentration-rate curve for the enzyme. As has been explained, this results in a sort of neutral equilibrium, the enzyme proportions corresponding to the trained state being preserved until some active agency intervenes and causes reversion.

In this model the linking of two enzymes by way of the intermediate substrate provides the mechanism of the adaptive change. An essential part is played by the response of enzyme II to changes in c. When c becomes so large that this response no longer occurs

† To avoid the introduction of complicated expressions into the rate equations, we have taken the rate of formation of the enzymes to be directly proportional to c and c' respectively. This is nearly correct for overall growth rates *below* the normal. If c and c' exceeded the normal values, for drug-free conditions the enzymes would become saturated and the rates show no increase. Equation (7) taken as it stands would imply a growth rate greater than K for trained cells tested at less than m. But it must only be applied for values up to K.

‡ D. S. Davies, A. M. James, and C. N. Hinshelwood, loc. cit.

we have, as it were, an uncoupling of the two enzymes. It will be useful to consider the conditions of growth of the uncoupled enzymes.

The equations (1) and (3) of p. 130 now become

$$dx_1/dt = k_1 x_1,$$
$$dx_2/dt = k_2' x_2$$

where k_2' is a constant slightly greater than $k_2 f(c_1)$.

It follows that

$$\frac{x_2}{x_1} = \frac{(x_2)_0}{(x_1)_0} e^{(k_2'-k_1)t}.$$

There can be no stable ratio of x_2/x_1 unless $k_2' = k_1$.

In the kind of case we have been considering hitherto the equality is ensured by the adjustability of c. If all enzymes were normally operating on the level part of their substrate concentration-rate curves, this would not be possible. Adaptive changes would then require actual modification of k_1 or k_2'. This would clearly require change in the molecular texture or in the configuration of the enzymes themselves.

Naturally, if substrates of altered composition are supplied to an enzyme, it may build up a somewhat changed protein texture, so that *qualitative* as well as *quantitative* changes in the enzymes cannot in principle be ruled out of consideration. This matter will be more appropriately discussed in connexion with adaptation to new sources of carbon or nitrogen. For the present we can take it that there is no objection to the supposition that c is normally near c_1 and that changes in the relative *quantitative* amounts of the enzymes play at least a very important part in adaptation.

According to this view, the trained cells in the normal medium are in a sort of metastable state. They possess a proportion of what we have schematized as enzyme I in excess of the normal, but have no active reason to lose it. In this connexion, the phenomenon of induced reversion is of importance. The loss of proflavine-adaptation of cells grown in presence of cresol has already been mentioned. Another interesting example is that of sulphonamide-adapted cells, which are deprived of their immunity by growth in presence of proflavine. That the action of the proflavine is exerted by way of an influence on certain relative rates is shown by the fact that once the cells have been trained to proflavine itself, they are no longer susceptible to its adverse effect on subsequently acquired sulphona-

mide-training. This would hardly be expected if the proflavine exerted some direct chemically destructive effect on an enzyme texture adapted to be insensitive to sulphonamide. The conditions under which the most effective proflavine de-training of sulphonamide-trained cells occurs are similar to those in which proflavine causes the formation of long filamentous cells, that is to say, conditions under which a maximum disturbance in the normal balance of cell processes is brought about.†

11. Joint action of two drugs

If, as has sometimes been thought, the slowest of the sequence of cell processes were rate-determining, then the joint effect of two drugs which interfere with the reaction series at two different stages would be simply equal to the greater of the two separate effects. If there is no cross adaptation, then we may conclude that two drugs do in fact intervene at different points. The matter in question can thus be tested. As might have been concluded from the discussion in IV. 4, the results show that neither of two separately exerted drug actions can be regarded as constituting a single bottleneck. *Bact lactis aerogenes* does not show cross adaptation to proflavine and sulphanilamide. The lags in various mixtures of these drugs were determined by A. M. James and compared with the lags caused by them singly. In all examples the lag in the mixture was found to be greater than that in either of the separate drugs. In more than half the experiments made, however, the lag in the mixture was less than the sum of the corresponding individual lags.

12. Influence of pH on the action of proflavine

The effect of pH on the bacteriostatic action of acridine derivatives with *Bact. coli* has been studied by Albert and others.‡ The drugs are much less effective in acid than in neutral solutions. The active molecular species is the positively charged acridinium ion. Over the range of pH in which the bacteriostatic action shows very great changes there is little change in the degree of ionization of the drug, which corresponds throughout to a great preponderance of the cationic form. The influence of the hydrogen ion concentration must be exerted, therefore, not on the drug itself, but on the proteins of

† A. M. James, unpublished results.

‡ A. Albert, S. D. Rubbo, R. J. Goldacre, M. E. Davey, and J. D. Stone, *Brit. J. Exp. Path.*, 1945, **26**, 160.

the cell. Albert supposes a competition between hydrogen ions and acridinium ions for various negative centres in the protein.

The following is a simple treatment of such an effect. Let the negative centres in the cell material be represented by X^-. Then for the acid dissociation we have

$$\frac{[X^-][H^+]}{[XH]} = K_1,$$

and for the combination of the protein material with the acridinium cations

$$\frac{[X^-][AH^+]}{[XAH]} = K_2.$$

Since nearly all the drug is present in solution as cation we may write $[AH^+] = m$. From the equations, we then find

$$\frac{[XH]}{[XAH]} = \left(\frac{K_2}{K_1}\right)\frac{[H^+]}{m}.$$

If [XH] be regarded as the active surface of the protein and [XAH] as the inactive surface, then the last expression is $s/(1-s)$, whence

$$s = \left(\frac{K_2}{K_1}\right)\frac{[H^+]}{m} \Big/ \left\{1 + \left(\frac{K_2}{K_1}\right)\frac{[H^+]}{m}\right\}.$$

It seems reasonable to assume that, for equal drug effects, s should be constant, which means that $[H^+]$ should vary linearly with m. In other words, the concentration of the drug required to produce a standard degree of inhibition should increase in direct proportion to the hydrogen ion concentration. Albert and collaborators† find for *Bact. coli* that a tenfold change in m corresponds to a shift of 1·2 pH units; instead of to 1·0 as the simple expression just derived would demand. A. R. Peacocke,‡ working with *Bact. lactis aerogenes*, in synthetic media finds m to vary with a power of the hydrogen ion concentration of about 0·7. This power does not change detectably when the cells have been previously adapted to moderately high concentrations of the drug (Fig. 48). The influence of the buffer salts on the dissociation constant of the protein and upon the adsorption of the acridine derivatives is hard to assess, so that perhaps quite simple relations are hardly to be expected.

† Loc. cit.
‡ Unpublished experiments.

13. Changes in enzymatic properties of cells brought about by adaptation to drugs

The fundamental assumption made in the preceding discussions of adaptation is that a change in the enzyme balance of the cells occurs during growth in the new environment. The question arises

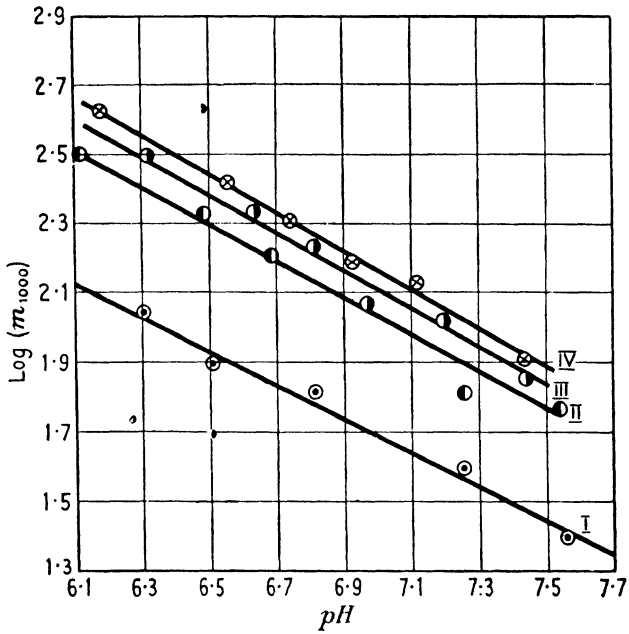


FIG. 48. Influence of pH on proflavine action.

m_{1000} is the concentration of proflavine required to increase the lag by 1,000 minutes.

I. Untrained cells. II. Cells trained to 21 mg./l. III. Cells trained to 44 mg./l. IV. Cells trained to 71 mg./l.

whether such changed enzyme balance is susceptible of direct demonstration—by methods other than inference from the altered growth rates themselves. Quite a number of suggestive observations are in fact on record. Smirnow† made various qualitative experiments on changes in fermentative properties of bacteria. Krüger‡ stated that the culture of *Bact. enteridis* (Gärtner) on agar containing malachite green gave rise to changes in the enzymes, but led to no permanent acquisition of new properties.

† M. R. Smirnow, *J. Bact.*, 1916, **1**, 385.
‡ H. Krüger, *Z. Bakt.*, 1938, **141**, 77.

Sulphapyridine-resistant strains of *Pneumococcus* are stated to have changed in their metabolic activities: peroxide and dehydrogenase are much reduced.† Variants have been obtained from *Pneumococcus*, under the influence of sulphonamides, showing a range of different metabolic activities—in forming hydrogen peroxide, fermenting inulin, and displaying different degrees of virulence.‡

A good deal of effort has been expended in discovering whether sulphonamide-trained bacteria produce increased supplies of the natural sulphonamide antagonist, para-aminobenzoic acid. The results differ from strain to strain, or, at any rate, according to the conditions of the experiments. Sulphathiazole-resistant strains of *Staphylococcus aureus* were stated to produce more than the parent strain from which they were derived.§ Housewright and Koser‖ found that sulphonamide-resistant *S. aureus* produced 10–1,000 times as much as the non-resistant parent strain, but that the trained strains of *Pneumococcus* and *Shigella paradysenteriae* showed no increase in *p*-aminobenzoic acid synthesis. Lemberg, Tandy, and Goldsworthy¶ found no increased production when a strain of *Bact. coli* was trained to lower concentrations of sulphathiazole, but an increase when higher concentrations were employed. They quote the results of other observers as revealing extra production with *Staphylococcus*, *Gonococcus*, and *Pneumococcus*, and no extra production with *Bact. coli*.

Bact. lactis aerogenes trained to moderate concentrations of sulphanilamide, proflavine, or crystal violet showed no significant changes in its dehydrogenase activity. When trained, however, to high concentrations of crystal violet the dehydrogenase activity towards glycerol was found to be much lowered, and this change was reflected in an increased difficulty of growth in glycerol media. Training to high concentrations of proflavine had the opposite effect.††

The induced loss of sulphonamide adaptation of *Bact. lactis aerogenes* when grown in presence of proflavine has already been referred to. In this connexion it is of interest that training to sulphonamide

† C. M. MacLeod, *Proc. Soc. Exp. Biol. Med.*, 1939, **41**, 215.

‡ R. A. McKinney and R. R. Mellon, *J. Inf. Dis.*, 1941, **68**, 233.

§ M. Landy, N. W. Larkum, E. J. Oswald, and F. Streightoff, *Science*, 1943, **97**, 295.

‖ R. D. Housewright and S. A. Koser, *J. Inf. Dis.*, 1944, **75**, 113.

¶ R. Lemberg, D. Tandy, and N. E. Goldsworthy, *Nature*, 1946, **157**, 103.

†† D. S. Davies, unpublished experiments.

enhances the catalase activity of the cells, while growth in presence of proflavine lowers it, both for normal cells and for cells which have previously been trained to sulphonamide.†

When repeatedly subcultured at a pH removed from the optimum value, *Bact. coli* does not show adaptation: the pH optimum and the limits of tolerance remain unchanged. The enzyme content of the cells, however, may show changes with the pH prevailing during growth.‡ One group of enzymes, including urease, and catalase, change in amount with the pH of growth in such a way that the effective activity remains constant. For example, if the pH is remote from the optimum, more enzyme is formed, the increased amount just compensating for the decreased activity at the adverse pH. This means that cells grown at an adverse pH and tested at a favourable one show a higher activity than those grown and tested at the favourable one.

With a second group of enzymes, including amino acid decarboxylases and deaminases, there is no such effect. The possibility revealed by the first group of a compensation whereby the amount of enzyme per cell increases until a given rate of action is restored is of the greatest interest from the point of view of the adaptive models discussed earlier in this chapter.

Further reference to the changes in enzyme activities accompanying adaptation will be dealt with in connexion with training to new sources of nutrient material.

† E. H. Cole, unpublished experiments.
‡ E. F. Gale and H. M. R. Epps, *Biochem. J.*, 1942, **36**, 600.

VII

ADAPTATION TO NEW SOURCES OF CARBON OR NITROGEN

1. Introduction

THE literature of bacteriology abounds in references to the adaptation of bacteria to new sources of nutrient material. Cells of a given species are frequently unable to utilize a given carbon or nitrogen source with maximum efficiency until they have, as it were, become acclimatized to it. Sometimes they appear initially unable to use it at all. The method of 'training' which must then be resorted to is as follows. The cells are first grown in a mixture of A, in which they grow readily, and B, to which they are to be trained. Then, in successive subcultures, the ratio of A to B is reduced until A is made vanishingly small and finally dispensed with altogether. For example, *Bact. coli mutabile* with glucose as carbon source will not initially use ammonium sulphate as its nitrogen source, but grows very readily with asparagine. After 10 subcultures in a medium with 25 mg. of ammonium sulphate and an amount of asparagine decreasing progressively from 10 mg. to 0·05 mg., the cells were found in an actual experiment to have become fully adapted to grow in the ammonium sulphate without the addition of any asparagine at all.

No attempt will be made in this section to examine historically the study of adaptation to new sources, or to assign priority for the essential experimental observations. We shall deal with these observations in the order in which they are most relevant to the argument as a whole.

A question, which incidentally is of very great importance for practical bacteriology, and one which has also its theoretical interest, is that of the extent to which adaptation can go. Methods of classification and identification of bacteria sometimes make use of biochemical criteria such as the ability to ferment lactose: if this were simply and solely a question of training, then such criteria would lose most of their value. Now the fact is that in some examples tests based upon carbohydrate utilization prove to be quite reliable (e.g. in the subdivisions of the coliform group), while in others they are quite erratic. One may therefore conclude that adaptation is capable

of modifying properties over a wide, but not indefinitely wide, range.†

2. Types of adaptive response

The quantitative study of the growth process reveals several different patterns of adaptive behaviour, which will be described in

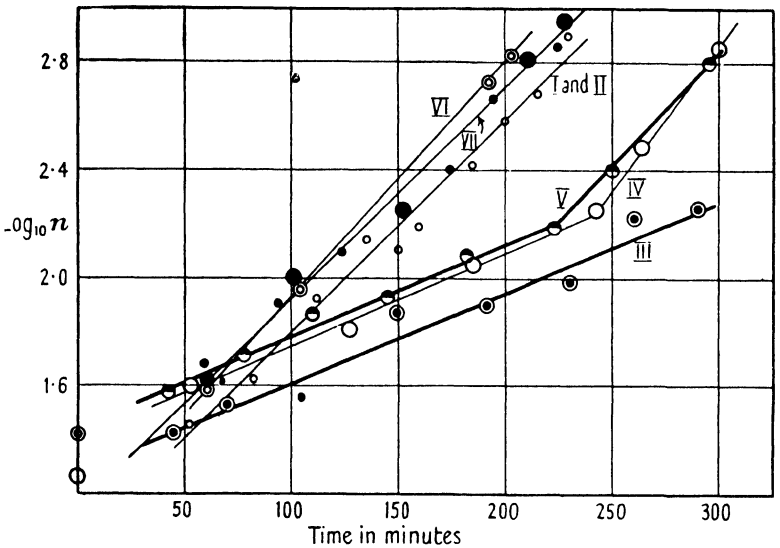


FIG. 49. Adaptation in glycerol medium and its reversal—typical growth curves.

I and II. Cells trained by 7 and 9 passages respectively. III. 9 training passages, 1 reversion passage. IV. 9 training passages, 2 reversion passages. V. 6 training passages, 2 reversion passages. VI. 9 training passages, 2 reversions, followed by 1 training passage. VII. 6 training passages, 2 reversions, followed by 3 training passages. The time origins are adjusted to avoid inconvenient overlapping of curves. (Note composite form of certain of the curves and compare with Fig. 35.)

terms of typical examples. Sometimes the main interest centres in the lag, sometimes in the generation time, sometimes in the actual form of the growth curve.

I. *Composite growth curves*

In this type of behaviour there is no specially long initial lag, but the growth curves assume the broken form shown in Fig. 49. In successive subcultures the transition from the slower to the faster mode of growth occurs at a progressively earlier stage. Analogous

† See *inter alia*, A. I. Virtanen, *J. Bact.*, 1934, **28**, 447.

behaviour has already been encountered in the example of the training of *Bact. lactis aerogenes* to resist sulphonamides.†

(*a*) Bact. lactis aerogenes *and glycerol.* An excellent example of this kind of behaviour is shown by the adaptation of *Bact. lactis aerogenes* to glycerol.‡ Typical growth curves obtained during the training of the cells to glycerol (after transfer from a corresponding medium containing glucose) are shown in Fig. 49.§

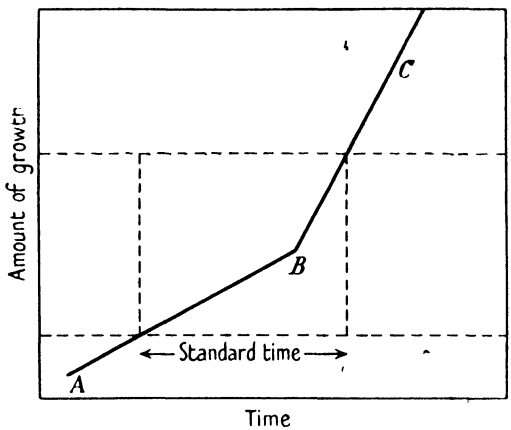


FIG. 50.

When the growth curve is of the form shown, the progress of adaptation cannot be represented by changes in the value of the mean generation time. In Fig. 50 for example, the adaptation might occur by a combination of three effects: an increase in the slope of AB, an increase in the slope of BC, or the downward movement of B. For a numerical measure the most convenient quantity to record is the *time* taken for the count to increase from a standard value well below B to another standard value well above B: any of the three effects are thereby included. The course of adaptation to glycerol in successive subcultures is shown in Fig. 51.

If the glycerol-trained cells are passed through a glycerol-free medium (containing glucose as carbon source) soon after they have reached the limiting degree of adaptation (maximum growth rate),

† See p. 122.

‡ R. M. Lodge and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1944, **40**, 571; E. G. Cooke and C. N. Hinshelwood, in the press.

§ All data refer to 40° C. unless otherwise stated.

a reversion occurs at about the same rate as the original adaptation. This reversion is revealed by periodic tests in glycerol as illustrated in Fig. 51. The final 'reverted' state is not necessarily identical, in respect of growth rate in glycerol, with the original, but different

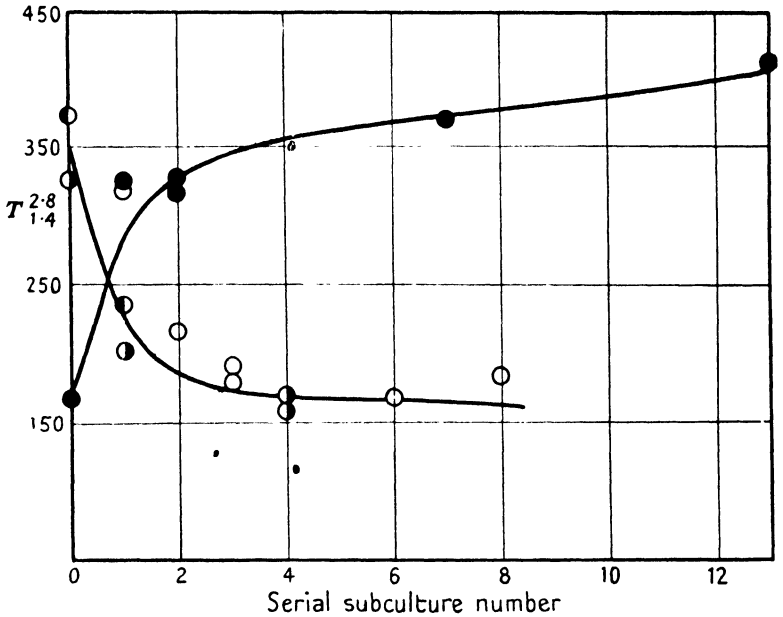


FIG. 51. Adaptation in glycerol medium and its reversal.

Passages through glycerol medium—falling curve: through glucose medium—rising curve (all measurements in glycerol medium).

Open circles—training: full circles—reversion: half-shaded circles—re-training after reversion. The number of training passages before the reversion tests was 6 or 9. (N.B. *Short* times signify *high* degree of adaptation to glycerol.)

original strains of the same species vary a good deal in this respect among themselves.

If the training to glycerol is very prolonged, the adapted strain shows no reversion even after 50 subcultures in the absence of glycerol, and may be taken to be stable. When the training has been carried through an intermediate number of subcultures, a phenomenon which may be termed *delayed reversion* is observable. On passage through the glycerol-free medium, the strain retains its full glycerol adaptation (as shown by separate tests in glycerol) for a certain number of subcultures and then rapidly reverts. The course

of events is shown in Fig. 52† and is exactly parallel with that found for lactose adaptation (see below).

(*b*) Bact. lactis aerogenes *and glycine*. This example resembles the previous one, except that the first segment of the growth curve is more sharply separated from the second. Growth by the first mode reaches its stationary phase before that by the second overtakes it. Actual growth curves‡ are shown in Fig. 53 and the relation between these and the curves of Fig. 49 is suggested by Fig. 54.

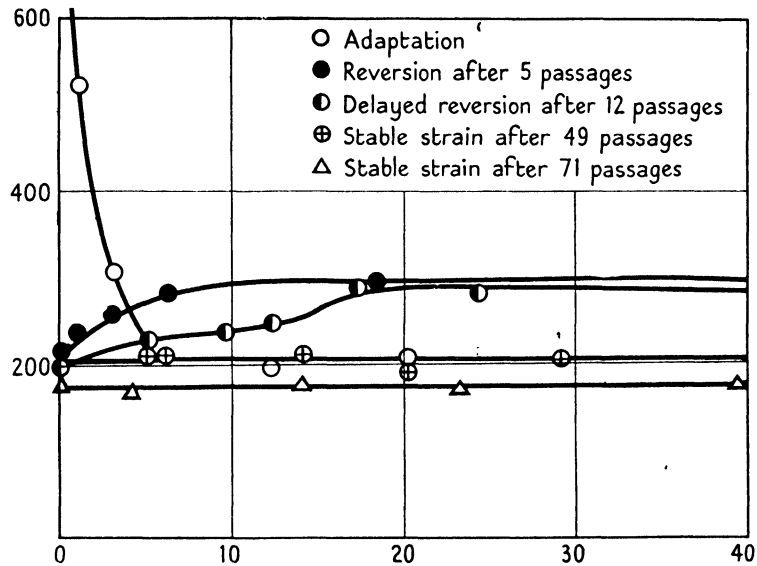


FIG. 52. Stages in glycerol-adaptation.
Immediate reversion: delayed reversion: non-reversion. (Compare Fig. 56 for lactose.)

In its earlier stages, the adaptation to glycine is reversible, but, from analogy with other cases, it probably becomes irreversible on long-continued training. The full stabilization was not observed experimentally but the training was probably not carried on for long enough for it to be established. A certain progress towards it was, however, detected after fifteen passages.

II. *Adaptation of mean generation time*

The break in the growth curves, as described under I, is not always in evidence, though it may in such examples have occurred before

† E. G. Cooke. ‡ R. M. Lodge and C. N. Hinshelwood, loc. cit.

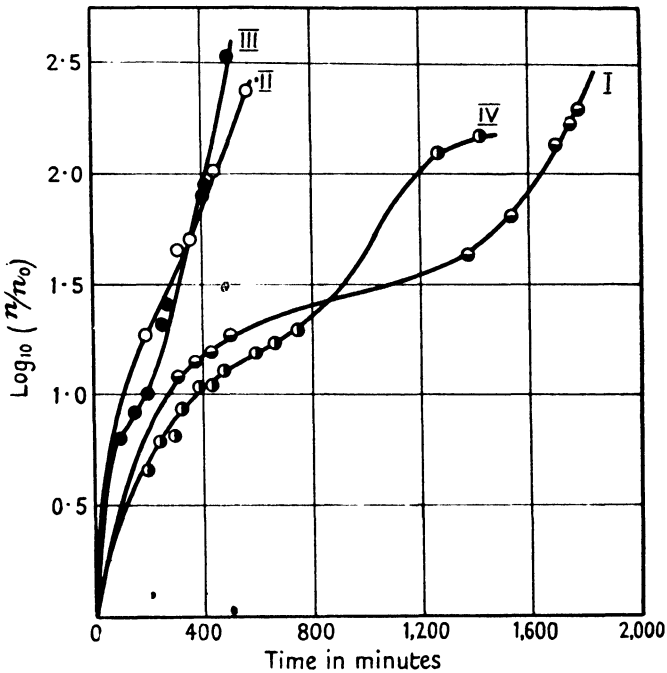


FIG. 53. Form of growth curves in glycine medium.

I. Untrained. II. Trained. III. Trained (washed cells). IV. After 9 reversion passages.

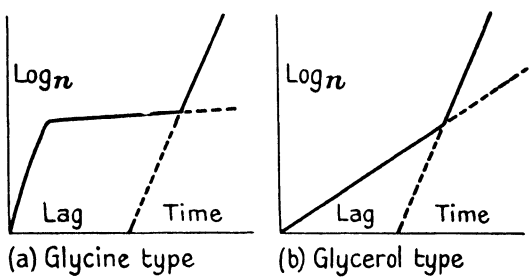


FIG. 54. Schematic, illustrating relation of glycine-type and glycerol-type growth curves.

the bacterial counts came into the range of convenient measurement. At all events in the usual range of measurement all that is observable is a progressive decrease in the mean generation time.

(*a*) Bact. lactis aerogenes *and lactose.*† There is no specially great lag when *Bact. lactis aerogenes* adapted to glucose is transferred to a corresponding lactose medium. On the first occasion it is about 6 hours longer than in a parallel subculture into glucose. On subsequent subcultures the difference vanishes: the lag may even become slightly less in the lactose medium. After a few subcultures the mean

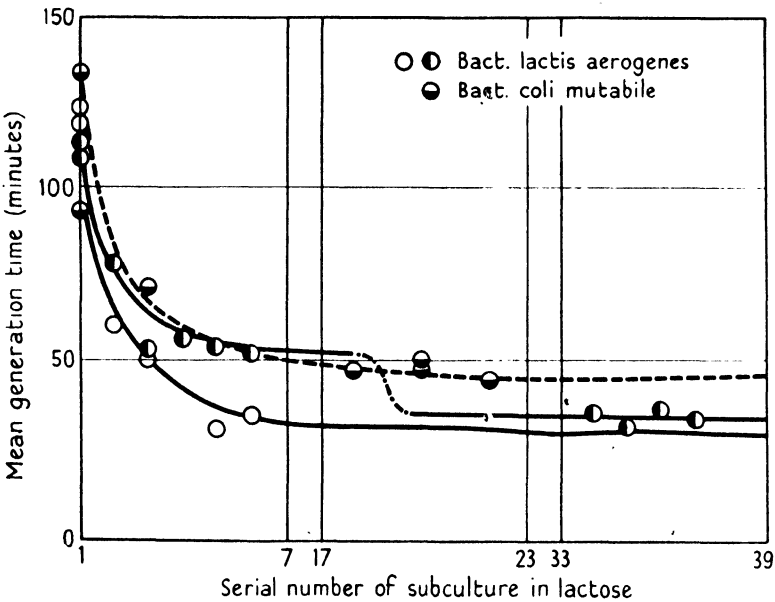


FIG. 55. Training to lactose.

generation times, initially about 100 minutes, attain the same value as in glucose, namely about 33 minutes. If the cells are removed from the lactose medium at this stage and returned to glucose, rapid reversion occurs. If transferred after a longer training in lactose, there may still be reversion, but it is not observable until after a delay of quite a number of subcultures, during which it appears to remain in suspense. Finally, after very thorough training, the lactose adaptation becomes stable enough to persist even after 30–40 subcultures in absence of any lactose. The training-reversion relations are shown in Figs. 55 and 56. The behaviour observed in connexion with training to glycerol is closely similar.

† J. R. Postgate and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1946, **42**, 45.

(*b*) Bact. lactis aerogenes *and other disaccharides.* The training of *Bact. lactis aerogenes* to maltose and sucrose conforms to the same scheme as the training to lactose. The adapted strains all grow with a mean generation time equal to that found in glucose. Cells trained to glucose are, for certain strains at least, found to be already adapted to cellobiose.

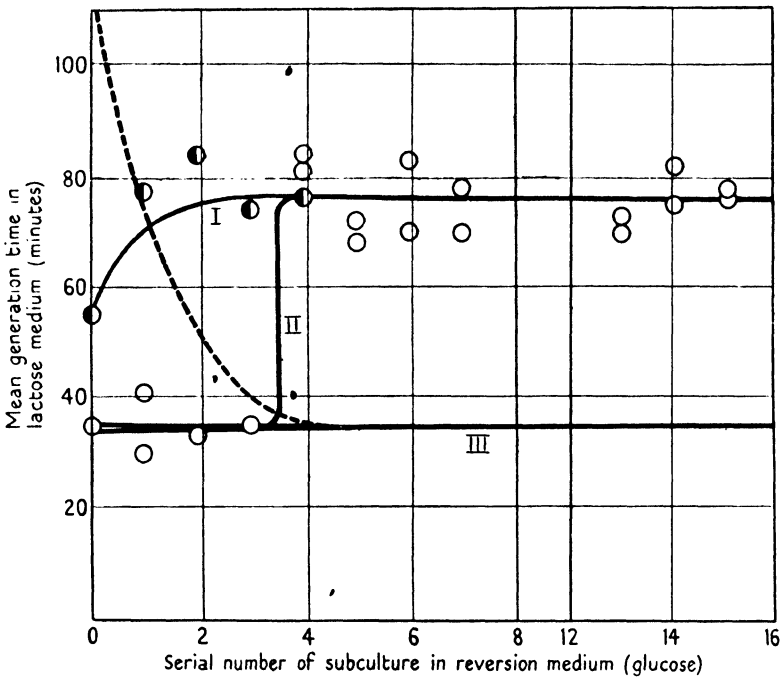


FIG. 56. Loss of lactose adaptation.

I. 5 training passages: rapid reversion. II. 31 training passages: delayed reversion. III. 100 training passages: no reversion. Dotted line shows curve of adaptation (cf. Fig. 55) for comparison.

Adaptation is not reciprocal, in the sense that cells trained to one of the three disaccharides, lactose (L-strain), maltose (M-strain), or sucrose (S-strain) are not adapted to the other two. By successive training, however, strains adapted to any pair (LM, MS, LS), or to all three (LMS), can be obtained from the original glucose-adapted strain. The relationships of the mean generation times are shown in the following table:

Strain trained to	Mean generation times (minutes) for strain tested in		
	lactose	maltose	sucrose
Glucose	100	110	60
Lactose	33	84	46
Maltose	77	32	42
Sucrose	97	109	33
Lactose + sucrose . .	33	..	31
Lactose + sucrose + maltose	31	35	34

Long continued serial subculture in lactose (and presumably in any of the other disaccharides) causes no loss in the capacity of the cells to grow with maximum efficiency in glucose.

It should be mentioned, however, that a lactose-trained strain, in the intermediate state where it is stable to 10 or 20—though not to an indefinite number—of subcultures in glucose, suffers a rapid induced reversion if it is grown in maltose.†

(c) Bact. lactis aerogenes *and* d-*xylose or* l-*arabinose*.‡ When transferred from a glucose medium to one containing *d*-xylose or *l*-arabinose, *Bact. lactis aerogenes* shows a nearly normal lag. The mean generation time is at first a little greater than that in glucose, but on subculture changes as a result of a rapid adaptation to a value differing little from it. The behaviour in *d*-arabinose is in very marked contrast; it is discussed under the next heading.

III. *Exceptionally long lag on first transfer*

This is a very characteristic and important case. It is closely related to the phenomenon described in § 4.

(*a*) Bact. coli mutabile *and lactose*.§ If *Bact. coli mutabile* is first trained to grow with maximum efficiency (minimum lag and mean generation time) in a synthetic medium containing glucose and ammonium sulphate, and identical inocula are then transferred simultaneously from a growing culture to a glucose medium and to a similar medium containing lactose instead of glucose, growth in the former occurs without lag. In the latter, however, the lag is about 48 hours, after which the growth is comparable with that in glucose. (In sucrose and cellobiose the strain refuses to grow at all.)

If from this first subculture in lactose further parallel transfers are made, it is found that the lag in lactose is never greater than that

† Cf. p. 191. ‡ E. G. Cooke, unpublished experiments.
§ J. R. Postgate and C. N. Hinshelwood, loc. cit.

in glucose and, for cells of suitable age, is zero. Thus in one subculture the lag has dropped from 48 hours to zero. Once the adaptation has occurred it is stable and no relapse occurs after many intervening subculturings in other media.

Lags of Bact. coli mutabile *in Lactose and in Glucose*

	Subculture	*Lag lactose — Lag glucose*
ex glucose into lactose or glucose	1	48 hours
	2	−1·5 hours
	3	−1 hour

		Lag lactose	*Lag glucose*
Lactose-trained, then 16 times through media without lactose. Then tested in lactose and glucose (ages various)	1	2·0 hours	3·0 hours
	2	44 hours	40 hours
	3	43 hours	40 hours
	(2 and 3—old inoculum)		

The changes in mean generation time are much less spectacular, and occur less rapidly. In the first few subcultures there is a tendency for the growth curve to show an irregular form, with an overall generation time of about 70–100 minutes. After 10–12 subcultures excellent logarithmic curves appear and the mean generation time settles down to a steady value of about 43 minutes which is the same as that found for growth in glucose.

The contrast between the behaviour of *Bact. coli mutabile* and that of *Bact. lactis aerogenes* is interesting. The magnitude of the adaptive effect is about the same for the two in so far as the mean generation time is concerned. But whereas the initial lag with *Bact. lactis aerogenes* is about 6 hours, with *Bact. coli mutabile* it is about 48 hours.

(*b*) Bact. lactis aerogenes *and* d-*arabinose*.† Untrained *Bact. lactis aerogenes* grows easily in *l*-arabinose: the lag-age curve of cells transferred from glucose showing a minimum value of the lag of practically zero. The behaviour in *d*-arabinose is in marked contrast: the lag on the first transfer of cells from glucose has a minimum value of approximately 60 hours (at 40° C.). As with *Bact. coli mutabile* and lactose, the second subculture shows a complete transformation of the picture: the lag-age curve now shows a minimum value of the lag of not more than about 3 hours.

The adaptation is stable in that numerous intervening passages through other media cause no reappearance of the long initial lag.

† E. G. Cooke, and C. N. Hinshelwood, in the press.

3. Failure of adaptive response

Sometimes attempts to evoke an adaptive response are quite negative. The following example illustrates a simple method of testing, based upon the training procedure of gradually replacing the old by the new substrate.

Bact. lactis aerogenes was inoculated simultaneously into two mixtures, one containing 200 mg. erythritol and x mg. of glucose, the other containing x mg. of glucose alone. In successive subcultures x was reduced. The total bacterial population n_s produced in each of the parallel cultures was measured. If there were no adaptation to the erythritol, n_s would drop in proportion to x, and would remain the same for the two tubes. If adaptation occurred, n_s would remain higher in the medium containing the erythritol. The numbers below show that the former alternative corresponds to the actual course of events.

mg. glucose	50	15	5	5	5	0
n_s . .	320	58	65	74	0	

In a similar experiment with arabinose the results were:

mg. arabinose .	25	10	2	2
n_s	++	++	+	83
Omitting erythritol .				97

Similar results were found for mixtures of erythritol with glycerol and lactose respectively. In the final tests of the series the counts were as follows:

Erythritol: glycerol	$n_s = 100$	Erythritol: lactose	$n_s = 204$
Glycerol alone .	$n_s = 88$	Lactose alone .	$n_s = 316$
Erythritol alone .	$n_s = 0$	Erythritol alone .	$n_s = 0$

4. The phenomena shown by *Bact. coli mutabile*

The classic example of what is usually termed variation was described by Massini† and has since been studied by numerous other workers. If *Bact. coli mutabile* is grown on a solid medium such as agar, it forms the usual colonies. These do not ferment lactose. If lactose and an indicator (neutral red) which turns red with acid are included in the medium, the colonies remain white, no acid having been produced by the fermentation of the sugar. If, however, the colonies are left for several days, daughter colonies of lactose-fermenting cells grow out from some of the original ones. The new ones are coloured red as a result of the fermentation. If any one of the lactose-fermenting colonies is removed and used to inoculate a fresh agar

† R. Massini, *Archiv f. Hyg.*, 1907, **61**, 250.

plate, all the derivative colonies retain the acquired property, which is hardly ever lost, whatever vicissitudes the cells may encounter before they are next given an opportunity of practising their powers on lactose itself. Massini, however, in his original paper, states quite definitely that on one occasion he observed a clear example of reversion, so that the new property is evidently not an absolutely inherent character of the 'variants'.†

Another example of the same sort of phenomenon is provided by certain strains of haemolytic *streptococci* which normally do not produce peroxide. When these are allowed to age on benzidine-blood agar for 4 to 10 days, they give secondary peroxide-producing colonies.‡ From these daughter colonies pure strains of peroxide-forming variants could be isolated.§

There is obviously a very close connexion between these formations of 'variant' daughter colonies and the kind of observation described in § 2 (III), where a given strain of cells refuses to grow in a certain medium except after an abnormally long lag, and where that lag is rapidly and practically irreversibly reduced to zero by adaptation. In the Massini phenomenon growth obviously occurs at the expense of other carbon sources than lactose: when these are exhausted growth stops, and is only resumed after an interval which can be regarded as the lag associated with the lactose utilization. Whether the lag as observed for the culture as a whole depends simply upon the time required for mutations to occur in certain cells, or whether it is the normal kind of lag shown by all cells not in the steady state, is a matter for further consideration. Since cells which have not previously used lactose lack necessary intermediates and have to develop a new reaction sequence, the latter explanation seems to be the simpler. The only difference between the formation of the lactose-utilizing variants of *Bact. coli mutabile* and the adaptation of *Bact. lactis aerogenes* to utilize lactose with maximum efficiency is one of degree.

The *mutabile* phenomenon does, however, focus attention clearly upon a fundamental question, namely, whether the adaptive process

† K. Baerthlein (*Centralbl. f. Bakt.*, 1912, **66**; 21) also records reversion of lactose fermenting to the non-fermenting types. 'Bei Züchtung auf Agar tritt dieser Rückschlag im allgemeinen bereits nach 6–8 Tagen ein.'

‡ F. P. Hadley, P. Hadley, and W. W. Leathen, *J. Inf. Dis.*, 1941, **68**, 264.

§ For a summary of various other observations, see A. Haddow, *Acta Int. Union against Cancer*, 1937, ii. 376.

is one which occurs to all the cells (even though the few in which it is complete first outstrip the others in the subsequent race), or whether it depends upon some highly specialized accident in very few cells. The practical difference between the two explanations is small—which makes experimental discrimination more difficult—but the principle is of great theoretical importance in relation to the mechanism by which adaptation is initiated. This whole question will be discussed from a more general standpoint in a later section, but it may be remarked here that the conception of the adaptive process as occurring in all the cells is the more consistent with the whole approach which has so far been explored in the present book.

5. Primary enzymatic changes accompanying adaptation to new carbon sources

The following results (D. S. Davies) show how clearly detectable are the changes in certain important enzymes after training to a new carbon source. *Bact. lactis aerogenes* was trained by serial subculture in media containing various carbohydrates. The dehydrogenase activity of washed suspensions of the cells (prepared by Quastel's method) was then determined with the aid of the Thunberg methylene blue technique. The following tables give (*a*) relative values for the growth rates for the various trained strains, (*b*) the dehydrogenase activities of these same strains towards the various carbohydrates. It will be seen that a close correlation exists, and that the optimum growth rate runs parallel with the optimum dehydrogenase activity.

Strain trained to glucose and to	*Relative growth rate in (Glucose = 100)*			
	Glucose	*Lactose*	*Maltose*	*Sucrose*
No other	100	33	30	55
Lactose	100	100	39	72
Maltose	88	43	103	79
Sucrose	106	34	30	100

Strain trained to glucose and to	*Relative dehydrogenase activity towards (Glucose = 100)*				
	Glucose	*Lactose*	*Maltose*	*Sucrose*	*Glycerol*
No other	100	0	131	47	..
Lactose	100	113	128	113	15
Maltose	100	0	115	44	27
Sucrose	100	7	100	106	..
Glycerol	100	15	..	..	100

The correspondence between the two sets of measurements is on the whole rather close. The chief anomaly is that the enzyme tests show a high activity towards maltose even of cells which have not been trained to grow optimally in this sugar. It would clearly be of interest to extend such results to include sources of nitrogen as well as sources of carbon.

E. G. Cooke† has shown that the whole picture (described in an earlier section of the present chapter) of the acquisition and loss of glycerol adaptation, as represented by growth rates, is generally reproduced, if glycerol dehydrogenase activity instead of mean generation time is taken as the criterion of the adaptation. On training of the cells the activity rises to a limit. If they are then subcultured in a glycerol-free medium, the extent to which reversion occurs depends upon the length of the original training. If the training has only just brought the activity to its maximum value, there will be a rapid decline. If it has been longer continued, there will be a slower and delayed decline. If the training has been prolonged then the activity of the enzyme remains high despite many subcultures in complete absence of glycerol.

6. Growth and adaptation

The results of the last section show that there is a correlation between training to give optimum growth in a given medium and certain enzyme activities in that medium. We must now inquire into the converse proposition: namely, how far the development of the optimum enzyme activity demands that there shall have been actual growth occurring in the presence of and accompanied by utilization of the appropriate substrate.

The first thing to note is that the activity of untrained cells towards a given new substrate varies over very wide ranges. Enzymes in bacteria have been classed by Dubos‡ as constitutive or adaptive, the latter being those which are only called into existence in presence of the appropriate substrate. Yudkin§ suggested reversible systems of enzymes and precursors of enzymes such that, if the enzyme is removed by combination with an inhibitor or otherwise, the equilibrium between it and the precursor is shifted in the direction of restoring the balance. As we have seen, *Bact. coli mutabile* possesses no initial

† Unpublished experiments.

‡ R. Dubos, *Bact. Rev.*, 1940, **4**, 1.

§ J. Yudkin, *Biol. Rev.*, 1938, **13**, 93.

power of utilizing lactose, while *Bact. lactis aerogenes* utilizes it from the start with quite high, though by no means optimal, efficiency.

Stephenson and Gale,† working with washed suspensions of *Bact. coli* concluded that power to ferment galactose was only acquired by cells which had actually multiplied in presence of this sugar. Stephenson and Yudkin,‡ on the other hand, concluded that adaptation of yeast cultures to galactose could occur without actual multiplication of the cells. Hegarty§ found that *Streptococcus lactis* in glucose-broth could not attack galactose, lactose, sucrose, or maltose unless it had been allowed to multiply in presence of the sugar in question. Knox and Pollock,|| studying the reduction of tetrathionate by *Bact. paratyphosum B*, found an initial induction period for the action of the washed suspension on the substrate. At the end of this period the reaction set in and the adaptation appeared to be complete. If cells which had acquired activity were centifuged and resuspended in tetrathionate, the reaction was rapid from the start; and the new property survived storage of the washed cells for long periods.

In the training of *Bact. lactis aerogenes* to glycine, referred to on p. 164, there is a clear indication that adaptation is closely linked with actual growth of the cells by that mode which involves the consumption of glycine. According to the interpretation of the growth curves discussed on p. 164, the mode of growth associated with the adaptation is that setting in after the arrest. Serial subcultures made in such a way that growth never proceeds beyond this arrest are attended with a very great delay in the training. After subcultures corresponding to 36 cell divisions there was no perceptible training: while, in a similar series of experiments where the subcultures were made well after the arrest, i.e. attended with considerable growth by the adapted mode, after 34 cell divisions the training had nearly reached the limit to which it could go.

The results, referred to on p. 112, on adaptation to proflavine, show that, here also, adaptation and growth in presence of the drug are closely connected.

In seeking to form a clear view of these various facts, one must

† M. Stephenson and E. F. Gale, *Biochem. J.*, 1937, **31**, 1311.
‡ M. Stephenson and J. Yudkin, ibid., 1936, **30**, 506.
§ C. P. Hegarty, *J. Bact.*, 1939, **37**, 145.
|| R. Knox and M. R. Pollock, *Biochem. J.*, 1944, **38**, 299.

distinguish between different senses in which the word adaptation might be employed. From one point of view, one might call the process occurring during the lag phase of an ordinary aged culture an adaptation. When first transferred to a fresh supply of its usual medium the culture is unable to grow: after a certain period growth sets in. If, however, growth goes to completion and the cells are allowed to attain the same age as in the previous experiment, a fresh subculture will lead to approximately the same lag as before. This we may contrast with what happens when drug-adaptation occurs: after a long initial lag, growth occurs, and if, after it is complete, the cells are allowed to attain the same age as in the previous trial and then are tested once more in the drug, this time the lag is much less than before. If the lag itself were called an adaptation time, then the process occurring during the growth in the drug would be an adaptation of a higher order, namely, an adaptation whereby less adaptation is needed on a subsequent occasion of the same kind. Now in the processes of training to grow in glycerol or to resist sulphonamides (pp. 162 and 121) it appears that on each successive subculture the lag of a new and more suitable mechanism decreases relatively to that of the original mechanism. This decrease in lag on successive subcultures must occur as a result of the changed enzyme balance established during the respective preceding periods of growth. As long as an actual increase in cell material occurs this change in balance is not only possible, but necessary, under the new conditions. It might be convenient to reserve the term adaptation for such growth-linked processes and to refer to other adjustments as lag or induction phenomena.

Such induction phenomena will arise from various causes: the necessity for building up, on any given occasion, the requisite intermediates; the necessity for the repair of the substance of individual parts of the cell without multiplication of the cell as a whole; the dependence of the function to be measured upon some chance contingency of events in the cell. The first two are already familiar in connexion with lag itself. The third will be encountered again at a later stage and in another relation. Yet another cause of delay is one familiar in the induction periods of ordinary chemical reactions, namely, the need for the removal of an inhibitor. Which factor may operate in any given example is a matter for detailed experiment.

7. Alternative modes of growth

One of the remarkable characteristics of bacteria is their power of utilizing the most diverse substrates and of bringing about the most varied chemical changes. If every substrate were attacked by a quite specific enzyme, the number of enzymes which would have to be assumed would be absurdly great. What must be postulated seems rather to be an array of functions of a rather general kind, capable of breaking down larger molecules into fairly simple standard fragments, which can then be incorporated in existing structures. If all the chemical reactions of the cell are resolved into consecutive stages of a rather primitive kind—addition of water, removal or addition of hydrogen, and so on—then the most complex operations can be brought about by the appropriate succession of these unit processes. The progress from the initial food materials to the final bacterial substance can then occur by a diversity of routes. For example, if an amino acid is supplied as a source of nitrogen, by one route the amino acid might first be deaminated and the ammonia used in further synthetic reactions: by another route, the amino acid might be incorporated as a whole and at a later stage suffer further transformations. The individual steps of the reactions by which breakdown of substrates and re-synthesis of cell material occur can be combined in a large number of ways, giving rise to what might be described as a whole network of routes. According to the particular substrates provided for the cell, the overall rate of formation of bacterial substance by any given route will vary widely. That combination of steps will in fact be used which gives the most rapid growth. This idea is expressed schematically in Fig. 57. With a given set of substrates growth may be most efficient by the route $ABCDE$. If one of the substrates is replaced by another, then it may happen that the step AB becomes difficult, and that a combination of reactions represented by the route $AFGHE$ is capable of giving more rapid overall increase of substance.

The proportions of the various bacterial enzymes which correspond to optimum growth by one route may not be—in fact could hardly be—the same as for another route. Consequently, we should expect cells which have been grown under conditions favouring one, to suffer a change in enzyme balance when transferred to conditions favouring another. The establishment of the changed balance will constitute an adaptation.

8. Changes of enzyme balance in new growth media

Adaptation to utilize new substrates differs at first sight from adaptation to resist antibacterial drugs: the latter appears as the quantitative recovery of an impaired function, while the former seems the replacement of one function by a qualitatively different one. On closer inspection, however, the distinction loses its sharpness. One of the ways in which adaptation to drugs occurs seems

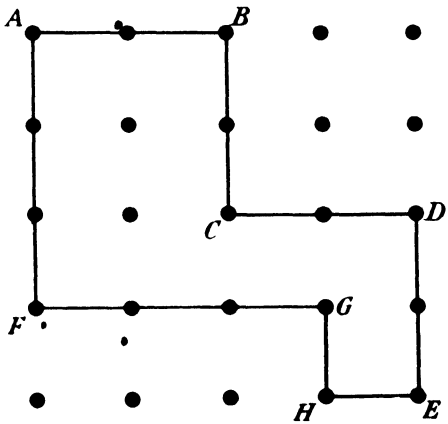


FIG. 57. Schematic. Alternative sequences.

to be by the mobilization of an alternative mechanism less sensitive than the normal one to the drug. Conversely, adaptation to the new substrate does not always involve the creation of an entirely new function, but, often, merely the improvement of an existing one. To take one of the examples described in the earlier part of the chapter, *Bact. lactis aerogenes* shows a quite marked adaptation to lactose, but, even cells which have not previously encountered this sugar are capable of moderately good growth in it. The adaptation simply involves the raising of an existing function to a higher pitch of efficiency.

In seeking possible explanations of the adaptive phenomena in terms of enzymatic changes, it will be convenient to consider in turn the three main types of response referred to in § 2. (This procedure does not, however, presuppose that they are necessarily dissimilar in nature.)

(1) *Increase of growth rate*

The easiest to begin with is that exemplified by the adaptation of *Bact. lactis aerogenes* to lactose. Here there is initially a moderate growth rate which, on serial subculture, improves rapidly to an optimum value. An enzyme capable of dealing with lactose is evidently present in the unadapted cells, and what occurs during the training process is either (*a*) the expansion of this existing enzyme, or (*b*) a qualitative modification in the structure of the original enzyme whereby it is enabled to utilize lactose with increased efficiency. Since our present concern is to explore general mechanisms we will examine these two possibilities in more detail.

(*a*) *Expansion of an existing enzyme.* In this case, no new function is acquired, though the capacity of an existing one improves on use. The enzyme capable of using the new substrate must therefore be formed even from the normal substrate. It must be regarded as a by-product of the normal functioning of the cell. (It might, for example, represent a certain special fraction of the surface of the enzyme dealing with the normal substrate.) We shall schematize this by assuming an enzyme j, the amount of which, x_j, is a constant fraction of the amount of enzyme 1, and which is capable of performing the function to be trained.

As on p. 130 we write the equations

$$\begin{aligned} dx_1/dt &= k_1 x_1, \\ n\,dc/dt &= k_1' x_1 - Kcn = 0, \\ dx_2/dt &= k_2 x_2 c, \\ n &= \beta x_2, \end{aligned}$$

where the symbols have the same meaning as before, and similar assumptions are made. We now add, however,

$$x_j = \gamma x_1.$$

It follows from these equations by integration that

$$x_j - (x_j)_0 = (\gamma\beta K k_1/k_2 k_1')\{x_2 - (x_2)_0\},$$

where $(x_j)_0$ and $(x_2)_0$ are the amounts present at time zero. On prolonged growth the enzyme ratios settle down to the value given by

$$(x_j) = (\gamma\beta K k_1/k_2 k_1')(x_2) = A(x_2), \qquad (1)$$

where (x_2) is the standard amount of enzyme 2 per cell.

When the cells are transferred to the new medium (e.g. with lactose

instead of glucose as a substrate), enzyme j, instead of being of secondary importance, becomes the seat of the key process. We now have it formed not as a by-product but from the new substrate according to the equation

$$dx_j/dt = k_j x_j.$$

Either directly or indirectly, its products lead to the synthesis of enzyme 2 as before. To simplify the equations we suppose this to occur directly (though the essential result does not depend upon this) and write

$$n\,dc_j/dt = k'_j x_j - K_j c_j n = 0,$$
$$dx_2/dt = k_2^* x_2 c_j,$$

whence, as before, we obtain the steady amount of x_j in a cell to be

$$x_j - (x_j)_0 = (\beta K_j k_j/k_2^* k'_j)\{x_2 - (x_2)_0\} = A_j\{x_2 - (x_2)_0\},$$

which, as soon as the new material outweighs the old, becomes

$$(x_j) = A_j(x_2). \tag{2}$$

The initial growth rate of the cells just transferred from the old medium will be $k_j A(x_2)$, while that of the adapted cells will be $k_j A_j(x_2)$ by equations (1) and (2) respectively. A may be as small as we please, since γ may be very small. The growth rate will then increase to a steady new value as enzyme j expands to its new proportion.

(*b*) *Qualitative modification in the structure of an enzyme.* In the last paragraph we supposed certain regions of the cell to be already capable of utilizing the new substrate with full qualitative efficiency. The adaptation consisted in their purely quantitative expansion. Another possibility is that the whole of the original enzyme, e.g. that dealing with glucose, is capable of dealing also with the new substrate, e.g. the lactose, but with a lower qualitative efficiency, calculated per unit amount of its substance. That is expressed by a reduction in the value of the constants k_1 and k'_1.

As was explained in I. 10, one can hardly escape the conclusion that the reproduction of living matter depends upon the orderly addition of like units to like. The new building blocks added at each stage must be cut to the shape and size which conforms to the existing pattern. This is analogous in many ways to the growth of crystalline matter by the addition of identical or isomorphous units. There will, according to this very general but none the less definite view, be

some geometrical relation between the enzyme and the substrate at the expense of which it increases its own substance.

Now crystals accept new material not only from their own chemical species, but from geometrically related species (oriented overgrowths, p. 16). In an analogous way we can imagine a glucose-utilizing enzyme incorporating into its texture elements derived from other carbohydrates. This process will probably occur rather less readily than the incorporation of units derived from glucose itself, and will be attended by some strain and distortion of the pattern. Nevertheless, provided that the disorganization is not too great, the slightly modified pattern will be built up on the old basis. The distortion requires extra activation energy and will be associated with a lower reaction rate. Hence the reduction in k_1 and k_1'. As the new structure increases, however, the original pattern becomes a smaller and smaller proportion of the whole, until eventually there is nothing left but modified pattern. From this, naturally, the strain has departed, and k_1 will have returned to a value which may be as great as, or for that matter, greater than the original. According to this view of the mechanism, the adaptation consists, not in the expansion of a given enzyme, but in the progressive replacement of its original texture by a slightly different one more closely conforming to the optimum geometrical relationship with the new substrate.

The processes envisaged under the two headings, (*a*) and (*b*), could, naturally, occur simultaneously. A new substrate might find a few specialized localities in the cell where it could be dealt with under appreciable distortion. As adaptation proceeds, the distortion is relieved by a modification of pattern, and at the same time the whole of the active area undergoes the requisite expansion.

(2) *Composite growth curves*

As has been explained, the curves in Fig. 50 can be formally interpreted in terms of two competing processes, one with a greater growth rate but initially longer lag, the other with a lower rate and shorter lag. The adaptation consists in the relative shortening of the lag of the former. If there were present in the system two quite independent groups of cells with the characteristics postulated, then such behaviour would be readily understandable. Indeed, at first sight, the not infrequent occurrence of composite growth curves might seem to lend support to the view that what we observe in

such examples is simply the outgrowing of one sub-species of bacteria by another sub-species in an initially inhomogeneous culture. As has been stated, it will be much more convenient to consider the part played by selection under one single heading. At the present juncture we shall consider what alternative explanations are possible.

We can account for a good deal by invoking jointly the principles of § 7 and those of (1) of the present section. We suppose that there exist two alternative reaction sequences, one favoured in presence of the original substrate, the other favoured in presence of the new substrate. The two substrates will be referred to as *A* and *B* and the two sequences as I and II respectively. Suppose growth has been occurring for a long time by sequence *A* with substrate I. The enzymes of sequence II are in fact built up as by-products (and may be, as stated before, simply specialized areas of other enzymes), but do not exist in any specially favourable proportions for the occurrence of sequence II. Some of them may, indeed, be present in very small amounts. If now the cells are transferred to substrate *B*, growth by sequence II, although chemically the optimum, may not be able to set in for some time, since the proportions of some of the essential enzymes may correspond to what would in effect be a very aged and decayed state had *B* been the usual medium. A long lag is therefore necessary before even the first cell division is ready to occur. If in the meantime no growth can occur by sequence I, all that we shall observe will be a long lag in the new medium followed by growth by sequence II. If, however, substrate *B* can be utilized at low efficiency by sequence I (as under *b* of the last section) then, since the enzymes for I are fully mobilized there will be no abnormal lag, but simply slow growth. The question now arises as to what happens if the two processes are combined. If the total bacterial substance can be slowly increasing by the inefficient combination (*B*, I) while the deficient enzymes of the more efficient process (*B*, II) are being built up to the required amounts, then we shall observe a growth curve with a transition point as in Fig. 50. The transition point will occur at the moment when the lag of the (*B*, II) process comes to an end.

The idea of a lag existing with respect to one process in a mass of matter increasing all the time by another is a rather difficult one. The following considerations seem, however, to show it to be reasonable. We suppose the bacterial mass to be increasing by the operation

of sequence I in presence of substrate B, the overall rate constant being k. Enzymes 1, 2,... are present at time t in total amounts $(x_1)_0 e^{kt}$, $(x_2)_0 e^{kt}$,.... The enzyme j, necessary for the initiation of the sequence II, is formed as a by-product in quite small amount. This we express by writing dx_j/dt with a term equal to $\gamma\, dx_1/dt$. As long as this is the only source of j, the amount per cell remains a fraction of the amount of 1. But in a medium containing substrate B, j is formed by direct synthesis from the substrate, the rate at time t being given by $k_j x_j$. Thus the total rate of production of j throughout the whole bacterial mass is given by the equation

$$\begin{aligned} dx_j/dt &= \gamma\, dx_1/dt + k_j x_j \\ &= \gamma k x_1 + k_j x_j \\ &= \gamma k (x_1)_0 e^{kt} + k_j x_j, \end{aligned}$$

or
$$dx_j/dt - k_j x_j = \gamma k (x_1)_0 e^{kt}.$$

This linear differential equation holds irrespective of the cell division. The solution is

$$x_j = (x_j)_0 e^{k_j t} + \frac{\gamma k (x_1)_0}{(k_i - k)} \{e^{k_j t} - e^{kt}\}.$$

When t is great enough, the first term on the right outweighs the second, since by the conditions of the problem k_j is greater than k, and γ is small. Eventually, therefore, we shall have an exponential rate of production of j with a rate constant k_j: and sequence II will take control. (What then happens to enzyme 1 is a matter for separate consideration.) But $(x_j)_0$ is very small compared with $(x_1)_0$. Therefore the amount per cell, $(x_j)_0/n$, is small compared with the standard amount which would normally provoke a division. Cell division is determined by x_1/n until x_j/n has passed a critical value given by $(x_j)/n$ where (x_j) is considerably greater than $(x_j)_0$. At this point growth by sequence II will supersede that by sequence I, and the overall rate constant k_j will replace that characteristic of the growth mode (I, B), namely k.

According to this view, growth proceeds by the relatively inefficient mechanism (I, B) during the time which is required for the small initial supply of enzyme $(x_j)_0$ to build up by an alternative process to the value (x_j). This can be properly termed the 'lag' of the alternative process even though n is increasing steadily. In the present sense the lag of the process II is the time required for x_j/n

to attain a threshold value. From the point of view of each cell, enzyme j starts at a very small value and attains the standard value at the end of the lag. That the number of cells has meanwhile been increasing is irrelevant, and merely represents a continuous change of scale of the phenomenon studied: it is as though we observed the induction period of a chemical reaction in a vessel which, as we watched, was filled fuller and fuller of the same reaction mixture.

As regards those parts of the cell substance which are built up by the joint action of enzymes 1 and j, their rate of formation will be approximately the sum of terms proportional respectively to dx_1/dt and dx_j/dt. While x_j is small this will be very nearly equivalent to saying that the total mass increases proportionally to e^{kt}, and subsequently, soon after x_j has passed the threshold, to $e^{k_j t}$. For the total mass, X, we may write approximately

$$X = ae^{kt} + be^{k_j t}$$

where k_j is greater than k but a is greater than b. In the usual way of plotting growth curves $\log X$ is expressed as a function of t. For small values of t the above equation gives a straight line of slope nearly equal to k, and in a certain region there is a rapid transition to a line of greater slope. Values of the function

$$100e^{t} + be^{3t}$$

are shown in Fig. 58 with $b = 10$, 1, and 0·1 respectively. The resemblance of the curves to those shown in Figs. 35, 49, and 50 is obvious.

(3) *Exceptionally long initial lag*

This phenomenon is exemplified by *Bact. coli mutabile* and lactose. There is no absolute need, as far as the explanation of the lag goes, to seek beyond the principles outlined in the previous paragraph. If $(x_j)_0$ were extremely small, and if, further, during the consequent lag of the sequence (II, B) the makeshift sequence (I, B) were unable to operate at all (that is, if the enzyme which normally deals with the substrate A were unable to deal with B at all), then the essential fact would be accounted for.

But this is the place to introduce the first reference to a principle which plays an important part in bacteriological discussions, namely, the possibility of the production of what are called 'variants'. When a cell divides, it might be supposed (and often is supposed) that

occasional abnormal distributions of matter occur in one of the daughter cells: and that these abnormalities lead to the appearance of special properties. If the properties which thus arise are favourable to growth in the medium in which the cell happens to be, then preferential multiplication of the privileged types must occur. According to this view, the long lag would be the time of waiting for favourable variations (plus that required for the variants to multiply).

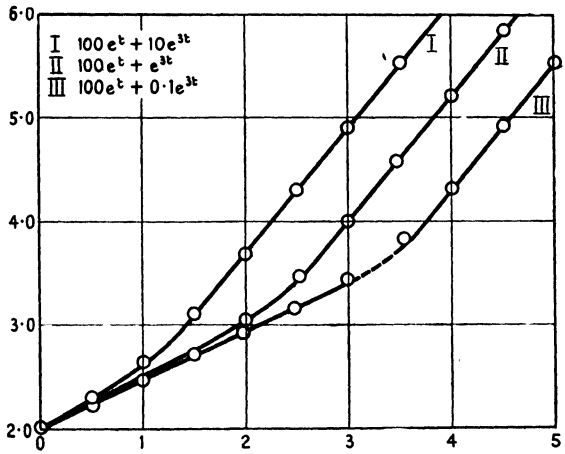


FIG. 58. Curves of function $100e^t + be^{3t}$.

Justice must be done to this vitally important principle in a later chapter.† Here it will only be observed that the idea of random variations seems better suited to explain the appearance of unexpected properties in cells which have been subjected to influences such as short-wave radiation, than to account for those examples where the properties acquired are nicely adjusted to the requirements of optimum growth in complex and specific ways. *Bact. lactis aerogenes*, as has been stated, can be trained to grow optimally in any combination of the sugars, lactose, sucrose, and maltose (taken singly or in pairs): it can acquire drug resistance precisely graded to the particular concentration of drug in which it has been grown. It is assigning a very heavy role to chance to attribute the initiation of all these adaptations to random variations. If we postulate an

† Cf. the discussion in A. Haddow, *Acta Int. Union against Cancer*, 1937, vol. ii, no. 4, p. 376.

abnormal mode of division as the origin of the modified strain, we can hardly escape the need for an auxiliary hypothesis explaining how the abnormal division can be guided by the presence of the specific substrate or of the drug. Such an hypothesis would probably have to have much in common with those which we have already explored in connexion with assumed changes in the cell material as a whole.

9. Reversion of adapted strains

The facts about adaptation and reversion, at first sight complex and confusing, seem on careful examination to conform to a general pattern. It appears that the following principles hold good both for adaptation to drugs and for adaptation to new substrates:

(1) Adaptation may be complete, in the sense of giving optimum growth rate in the new medium, without being stable against reversion when the cells are re-transferred to the original medium.

(2) The longer the training process is continued, the greater the stability. In some examples (possibly in all), a state is eventually reached in which the adaptation survives a hundred or more passages through media containing none of the substances by which it was originally provoked. There is sometimes an intermediate condition from which a delayed reversion is observable. The trained cells retain their adaptation for, say, 5 or 10 passages through another medium without any visible loss of training, and then on the next passage or two rapidly revert.

(3) Reversion may be partial in the sense that the reverted cells retain more of the acquired property than the original untrained cells possessed.

(4) The 'stability' of the most thoroughly adapted cells is not absolute. Reversion may sometimes be actively induced by growth under special conditions (e.g. destruction of sulphonamide training by growth in proflavine, p. 124), and may sometimes occur 'spontaneously', that is, from unknown or random causes (e.g. the reversion of the lactose variant of *Bact. coli mutabile* mentioned by Massini).

(5) Training usually imparts the new character without impairing the old. Cells which have been cultured normally in glucose may be thoroughly trained to utilize other carbon sources. The adapted cells are found, however, to have lost none of their power of growing

optimally in glucose itself. In other words, the adaptation represents an addition rather than a replacement. This rule is not invariable, but is common.

The facts summarized under the above headings (1) to (5) will now be considered in the light of the discussion of the previous section.

The hypothesis of enzyme expansion and that of enzyme modification would both predict complete reversal of the adaptation to a new substrate when the latter is again replaced by the original, except on one condition. That condition is that the adapted enzyme system is just as efficient as the original in reproducing itself by utilizing the old substrate. For example, the cell at first uses glucose optimally but not glycerol: it suffers expansion or distortion of part of its enzyme system so that glycerol is used optimally. This changed enzyme balance or texture must be preserved when glucose instead of glycerol is provided once more as the substrate. On this condition alone will there be no reversion. The fact that the adapted cells do at any rate grow with unimpaired efficiency (as measured by growth rate) in the old substrate suggests that the condition may well be fulfilled, and thus indicates a proximate reason for the absence of reversion. If the idea referred to above—namely that a glycerol or lactose utilizing enzyme is simply a specialized region of a glucose-utilizing enzyme—were correct, then one could see how an expansion of the specialized part would add the glycerol or lactose-utilizing character without impairing the 'glucose' character.

According to the argument as developed so far, we can envisage two cases: if the A-trained cells become $(A+B)$-trained on subculture in presence of B and if the A-type enzyme can be built up from B as well as from A, then there will be no reversion: if the B-type enzyme cannot be built up equally well from A then there should be reversion.

But the facts are more complex than this allows for. We have to explain why the adaptation should be initially unstable and should then, on continued training, acquire its stable, irreversible character. Of this fact, too, an explanation based upon crystallographic analogies could be suggested.

Suppose that the original enzyme has a certain texture adapted to substrate A, but one which can be built up from substrate B with production of a slightly distorted pattern. The new texture may also deal with substrate A and be capable of being built up from it. In

the early stages of the training to *B*, the old unmodified pattern and the new one are present together. Now we can well imagine that there is a very slight preference on the part of substrate *A* to build the original pattern *provided that there is a groundwork of it on which to build*. Thus if there is a mixture of new and old, the old will regain its preponderance in presence of the original substrate. On long-continued training, however, the bacterial substance is renewed again and again, and the vestiges of the unmodified pattern disappear completely. There is now nothing to guide the formation of the old *A*-type, and since *A* can build up the modified type easily enough, it continues to do so indefinitely. The 'delayed reversion' phenomenon will manifest itself just at the point where the original pattern has nearly but not quite disappeared from the cell. At first its reappearance will be negligibly slow, but once an appreciable amount has been formed it may help its own growth by a process analogous to the well-known co-operative effect in phase changes. The more the original type of site is restored, the more easily will the remaining sites change their texture to conform. According to the picture of a changing enzyme texture which accompanies adaptation, the progress through easy reversion, and delayed reversion to stability would be entirely understandable, and indeed the facts about reversion seem to constitute rather a good argument for some such view.

According to the hypothesis of changed enzyme proportions (rather than changed texture) the graded ease of reversion has to be attributed to the influence of secondary disturbances in the cell. A considerable expansion of an enzyme occupying an organized location in the cell can hardly occur without setting up strains in the structure of neighbouring parts. These will gradually be eased out as the cell material reproduces itself again and again, so that what was at first a source of instability in the new order gradually ceases to be so.

According to the hypothesis of spontaneous variations,† we should have to suppose that, by chance, there arises in the cell some molecular configuration upon which growth by utilization of the new substrate can proceed. We still have to accept the fact that the new kind of enzyme can continue to be built up from the old substrate, unless we interpret the facts of reversion entirely in terms of a changing population balance (see the discussion of selection, Chapter IX).

† See W. J. Penfold, *J. Path. Bact.*, 1910, **14**, 406.

10. Loss of properties

When cells, under the influence of changed substrate conditions, grow by new enzyme sequences, one might expect existing properties occasionally to be entirely lost. If a given part of the cell material is not enabled to grow with the overall rate constant k, but only with a smaller one k', then the proportion of the latter material diminishes progressively in proportion to $e^{-(k-k')t}$, and on continued subculture will become vanishingly small. Thus one might expect complete loss of properties to be commoner than it in fact is. Loss of particular characters does indeed occur. For example, when *Bact. coli* was exposed to X-rays it gave variants among which were some which had lost the power of growth without the specific addition to the medium of previously unnecessary growth factors. Similar results were found with *Acetobacter melanogenum* which gave variants requiring respectively glycine, leucine, or other specific amino acids.† By the action of X-rays, however, one can imagine the actual destruction of certain parts of the cell substance. Such results are not commonly found in connexion with simple training experiments. Drug action may repress enzyme activity, but does not usually eliminate it in such a way that it cannot be restored by re-training. *Bact. lactis aerogenes* was found to lose much of its glycerol dehydrogenase activity on training to high concentrations of crystal violet, but easily regained it on subculture in presence of glycerol.‡ It would seem, then, necessary to postulate the existence of certain kinds of site in the cell which, without being enzymes themselves, serve as ineradicable groundworks on which enzymes can be built.

The observations of Spiegelman and Lindegren§ on yeast cells have a close bearing on this matter. Some yeast cells are able to ferment melibiose, and thus contain melibiozymase. When these cells are placed in a medium containing melibiose the amount of enzyme increases, and when they are transferred to another medium the enzyme disappears. The process is stated to be reversible (though in the light of the experiments on intensive training of *Bact. lactis aerogenes* to lactose and to glycerol it would be interesting to know whether the adaptation might not become fully stabilized on *long-*

† C. H. Gray and E. L. Tatum, *Proc. Nat. Acad. Sci.*, 1944, **30**, 404.

‡ D. S. Davies and C. N. Hinshelwood, in the press.

§ *Ann. Missouri Botanic Gardens*, **2**, no. 2, 1945. Quoted among other relevant observations by G. Pontecorvo, *Nature*, 1946, **157**, 95.

continued growth in presence of the sugar). Unlike bacteria, yeasts can be crossed, and by appropriate crossings a strain could be obtained which possessed the melibiozymase and could maintain it as long as it was cultured in presence of the sugar. As soon, however, as it was grown in the absence of melibiose, the enzyme was irrecoverably lost. What has happened here, is that a strain of yeast lacking the necessary fundamental pattern for the building of the enzyme inherited some of the enzyme material from another strain by the crossing process. As long as autosynthesis of this enzyme occurred all was well, but once it was allowed to decay there was no basal pattern on which it could be rebuilt. Some sites in the cell, although not themselves constituting the enzyme, are capable of being developed into it: others are not. This is of interest in connexion with the discussion in § 8 about the development of the enzymes for dealing with alternative substrates.

11. Training by reduction of concentration in a mixed substrate

This process has already been described. It is of considerable theoretical interest and would well repay much more detailed study.

The problem in its simplest terms is the following: cells will grow in substrate *A*, but are unable to utilize substrate *B*. If grown in *A* alone they do not form structures capable of dealing with *B*: in *B* alone nothing at all occurs. Yet when growing in presence of *A* they are able to enter into some sort of relation with *B* which makes its subsequent use possible even in the absence of *A*.

Evidently some active intermediate formed during the utilization of *A* can react with *B* to build up in the cells the enzyme required for the subsequent attack on *B* itself. To investigate which members of the *A* class can be successfully combined in training experiments with which members of the *B* class would be likely to throw light upon the nature of the intermediate states.

The whole phenomenon stands in a suggestive relation to the idea expressed in an earlier section that the cell reactions are in general resolved into sequences of rather simple steps, some of which can be made to serve a multiple purpose.

12. The phenomenon of 'diauxie'†

Monod has studied the growth of bacteria in mixtures of two carbohydrates. The conditions of his experiments were so chosen that

† J. Monod, *La Croissance des Cultures Bactériennes*, Paris, 1942; *Annales de l'Institut Pasteur*, 1942, **68**, 548.

exhaustion of the carbohydrate was the factor limiting growth (see p. 62). In these circumstances growth curves are frequently obtained which show two separate bursts of growth with an intervening arrest. Some typical examples are shown diagrammatically in Figs. 59 and 60.

It can be shown that the two parts of the curve correspond to the *successive* utilization of the two sugars respectively. The first sugar is

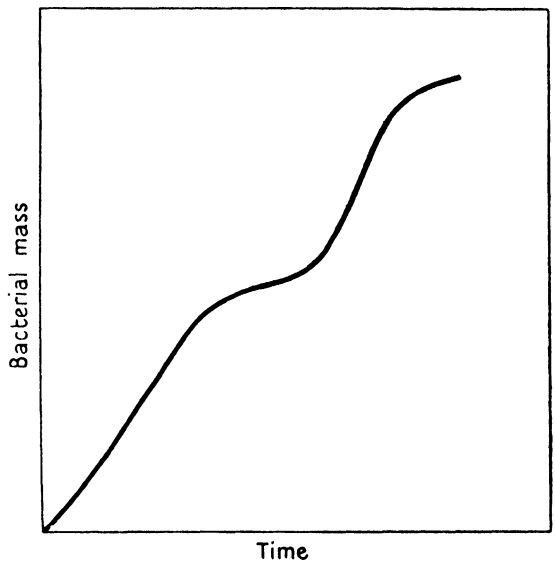


FIG. 59. 'Diauxie'.

all used up before the consumption of the second begins. At first sight it might seem that the bacteria are adapted to the sugar in which growth occurs first, but not adapted to the second sugar, so that the arrest occurs when the first sugar is consumed and the utilization of the second is still lagging. This view of the matter, however, proves to be inadequate. Preliminary training of the culture to the sugar in which the growth lags does not remove the arrest from the next curve determined for the mixture. Nor can two sugars which in combination do not normally show the double growth effect be induced to do so by the special training of the bacteria to one member of the pair. All the sugars, however, which show the delayed susceptibility to attack belong to the class for which adaptation of the bacteria is necessary. What is shown to happen is that the first

sugar actively inhibits the adaptation of the cells to the second, or even reverses it when once acquired.

These facts are closely related to the induced loss of adaptation to lactose which occurs when partially trained *Bact. lactis aerogenes* is cultivated in presence of maltose (p. 168). The growth in presence of the second carbohydrate brings about a loss of adaptation which

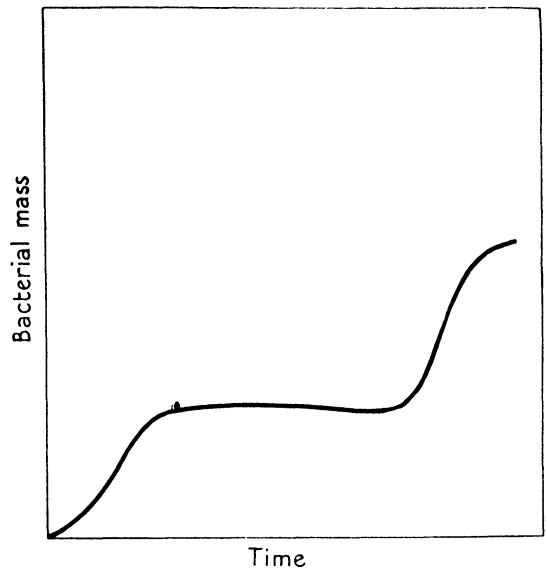


FIG. 60. 'Diauxie'.

even culture in presence of quite powerful antibacterial agents such as acridine derivatives or sulphonamides is unable to effect.

One noteworthy fact is that where violent means of inducing loss of adaptation fail, success may be achieved by the subtler one of allowing growth to occur in presence of a substance not very dissimilar to the one provoking the original training. Growth in presence of another carbohydrate exerts just the right disturbance on the adapted enzymes to provoke the reversion.

13. Comparison between the processes of adaptation to drugs and new substrates respectively

In many respects these two kinds of adaptation show very definite similarity. Rates of training are comparable, and in particular the

characteristic pattern of the adaptation-reversion relationships seems to be identical for both, as described in § 9.

The possible mechanisms by which the two types of process occur do not, however, completely correspond.

The primary action of a drug is to inhibit the growth of some parts of the bacterial substance, thereby causing a relative increase of other parts. The effect of a new substrate is to stimulate a relative increase of certain enzymes or a qualitative change in their actual texture.

For the stability of the adaptation to a drug, it is enough that the increased output of intermediates from the expanded enzymes should not, when the drug is no longer acting, cause a fresh readjustment. This condition can be fulfilled when the relation between reaction rate and intermediate concentration is of the quite plausible kind discussed in Chapter VI.

For the adaptation to the new substrate to be stable, it is necessary that the substance of the expanded or modified enzyme should be constructable from the original substrates.

In either case, there occurs the phenomenon whereby an unstably adapted culture reverts, not to the untrained state, but to an intermediate condition in which it retains a degree of training lower than that to which it was first subjected. If there has been a change in the texture of the enzymes, and nothing more, one would expect the modified form to be either completely stable or completely unstable according to whether or not any of the original form remained. If there has also been an expansion in the amounts of certain enzymes, it is reasonable enough to suppose that the resulting distortion of the general cell configuration can be tolerated up to a definite point but not beyond. This would mean that a moderate expansion would be stable while a greater degree would be unstable. The limit of toleration would, however, be a function of the training of the cell as a whole. And in this connexion a rather limited experience suggests that 'stable levels' are in fact more in evidence in connexion with drug adaptation than in connexion with adaptation to new substrates.

There is room for much more careful experiment before the whole story becomes entirely clear. The discussions of this chapter and the last leave many questions unanswered. But they seem to come near enough to the answers to enough questions to encourage further inquiry.

VIII

VARIANTS

1. Introduction

In the foregoing chapters bacterial adaptation, whether occurring in response to a change of medium or as a reaction to inhibition by drugs, has been regarded as an automatic adjustment of the enzyme balance. From this point of view, adaptation is called forth by the environment of the cells during growth. Many facts can be accounted for satisfactorily, even if not exclusively, in this way. It will now be convenient to consider various experiments and observations which have often been interpreted in terms of a different hypothesis, namely, that at the moment of cell division abnormal variants, possessing modified or even quite new properties, may arise. If such variants happen to be better adapted to grow in a given medium, then selection will naturally operate and they will outgrow and eventually replace their less well-endowed competitors.

The broad distinction is between, on the one hand, an actual directive influence of the environment itself on the constitution of the bacterial substance formed and, on the other hand, the existence of multiple possibilities which present themselves spontaneously at the moment of division, and upon which selection can operate if they happen to be favourable in the actual environment. Some writers on bacteriology have stressed the influence of environment in determining variations (for example, Flynn and Rettger,† and Gillespie and Rettger‡), while others have stressed the inherent power of cells to give new forms on division (for example, Bunting,§ Lewis,‖ Deskowitz,¶ and Parr and Robbins††).

It should be emphasized at the outset that one has not necessarily to do here with two competing or mutually exclusive hypotheses. Both types of mechanism may in fact operate in nature. The real problem is probably to determine correctly what is the precise role of each mechanism, and what is the relation between the two.

† C. S. Flynn and L. F. Rettger, *J. Bact.*, 1934, **28**, 1.
‡ H. B. Gillespie and L. F. Rettger, ibid., 1939, **38**, 41.
§ M. I. Bunting, ibid., 1940, **40**, 57, 69; 1942, **43**, 593.
‖ I. M. Lewis, ibid., 1934, **28**, 619.
¶ M. W. Deskowitz, ibid., 1937, **33**, 349.
†† L. W. Parr and M. L. Robbins, ibid., 1942, **43**, 66.

Moreover, in seeking a synthetic view of all the observations, a solution of the easier problem of what changes in bacterial substance growth in a given medium might evoke, should be of assistance in solving the more difficult one of what spontaneous changes would be necessary and how they might come about.

2. Some examples of variants

The observations of Massini on *Bact. coli mutabile* have already been described.† Lewis‡ showed that when this organism is grown on agar containing glucose the colonies invariably contain some variant cells capable of using lactose. By examining numbers of colonies grown on glucose, Lewis showed that all of them contained some proportion of the variants, as determined by viable counts with lactose media. From this he concluded that the original strain was not a mixture of the normal and the variant forms, but that the variants were thrown off during the growth of each separate colony. He also inferred that the variation was not called forth by the lactose.

This last conclusion merits careful analysis. What is found experimentally is, in principle, the following. From any colony grown in the absence of lactose two lots of N cells may be sampled (by suspension and dilution): one lot is plated out on to a solid medium containing glucose and the other lot on to a corresponding medium with lactose. Of the former, n_1 yield colonies and of the latter n_2. The fraction n_1/N for glucose approaches unity: that for lactose, n_2/N, is small, but not zero. Until the n_2 colonies actually grow there is no evidence that the cells from which they spring are lactose-users, and it is therefore, strictly speaking, difficult to be certain that they do not acquire their character after transfer to the lactose medium. The argument rests not so much upon the cells which do grow as upon those which do not. If all cells are capable of adapting themselves, given time, to lactose, then one must ask why n_2 is so much smaller than n_1. The answer may well be that the variation must have occurred before the transfer, and that all the cells transferred have not in fact the lactose-utilizing power.

This answer is, however, not quite unambiguous. Suppose, as an alternative, that adaptation does occur after the transfer to lactose: it may well entail a very long lag which only a few of the youngest

† VII. 4. ‡ I. M. Lewis, *J. Bact.*, 1934, **28**, 619.

and most vigorous cells in the inoculum survive, the others dying off. In this way one could explain the small yield of viable colonies obtained on the first transfer to the lactose agar.

More experiments on this interesting matter are obviously very desirable. In the first place, we need some quantitative measurements to show whether the apparent variants grow from the start with their optimum growth rate in lactose or whether they adapt themselves progressively. In the second place, it would be helpful to know whether those cells which have not proved viable on lactose at the time when the count of lactose-variants is taken are still alive and capable of growth if glucose is added to the medium.

For the time being, one is inclined to accept the argument of Lewis as probable, and to entertain the idea that when cell division occurs in absence of lactose, some few cells are more endowed than the others with the capacity to receive lactose adaptation. Nevertheless, the experiments on lactose adaptation described in Chapter VII show that actual growth in presence of the sugar plays an important role in developing, if not in initiating the adaptation.

Another important series of observations were made by Deskowitz,† in connexion with the colony forms of *Salmonella aertrycke*. When this is plated out in appropriate conditions normal and variant colony forms appear. If the latter are subcultured, they give, not a single kind of progeny but, once more, a mixture of types. The ratio of the new type to the parent type remains constant on repeated subculture. Change in the medium may cause an alteration in the ratio, but return to the original medium restores this to its original value. Deskowitz concludes that 'the potentiality to produce variants is an inherent property of the bacterial protoplasm independent of environment', and that a variant form will be thrown off once in a given number of cell divisions.

Similar conclusions were reached by Bunting,‡ who studied the behaviour of the organism *Serratia marcescens* (also known as *Chromobacterium prodigiosum*). This bacterium is well known for its capacity to produce a red pigment in appropriate circumstances. It was grown in a buffered ammonium citrate-glycerol medium and plated out, when it gave rise to dark red, bright pink, pale pink, and

† M. W. Deskowitz, ibid., 1937, **33**, 349.

‡ M. I. Bunting, ibid., 1940, **40**, 57, 69; 1942, **43**, 593; M. I. Bunting and L. J. Ingraham, ibid., 1942, **43**, 585.

white colonies, each of which was deemed to arise from a particular variant type. Re-plating from a colony of one particular type always gave more than one of the types as daughter colonies, which shows that there is no question of a mere mixture of stable strains, but some kind of dynamic equilibrium. If the bacteria were kept multiplying logarithmically in their liquid medium, and plated out from time to time, a definite steady state was reached in which 97 per cent. of the colonies would be 'dark red' and 3 per cent. 'bright pink'. There was no indication that any of the variants differed from one another in multiplication rate. Bunting considered that the phenomena observed were the 'expression of the probability of the occurrence of the specific intracellular events which are favourable to the production of variants'. As a culture in broth aged, the predominant population type showed a regular change. In the logarithmic growth phase the dark red colony-forming type was in excess, but at greater ages the pink and white types could increase to as much as 50 per cent.

The same general theme appears in the work of Parr and Robbins† on the utilization of citrate as sole carbon source by coliform bacteria. *Aerobacter* uses citrate at once: *Escherichia* not at all. Some coliforms use it only after a very long delay. This is interpreted to mean that any cell numbers among its progeny a few daughter cells which have undergone variation in the required sense. Some of the variants are stable, some revert easily. These observations on stability must, however, be considered in the light of the progressive stabilization of drug-adapted or medium-adapted cells on continued training (see VII. 9). Parr and Robbins in the discussion of their results on variation speak in terms of the 'mosaics of potentiality' in the cell: and of the 'reshuffle' of these.

3. Bacterial dissociation

The phenomenon called '*dissociation*' is a particular manifestation of variation affecting the forms of the colonies in which bacteria grow on solid media. It is so called because a single homogeneous strain gives rise to two or more types differing in character. It is perhaps a little unfortunate that so much of the literature deals with this particular aspect of variation, because we do not know at all well what other properties of the cells determine the colony form, and, in any event, growth in colonies is inevitably associated with

† L. W. Parr and M. L. Robbins, *J. Bact.*, 1942, **43**, 66.

overcrowding, exhaustion of foodstuff, and intense accumulation of toxic products. The chief forms of colony distinguished are smooth, rough (see p. 30), and mucoid. It seems that rough colonies are very often associated with larger cells or even filaments.†

A given bacterial strain may give colonies of a single type, smooth (*S*) or rough (*R*), or may give a mixture of forms. The *S* and *R* forms may be separated and subcultured: they may breed true, *S* giving *S* and *R* giving *R*, or revert. The trend of the so-called dissociation is influenced by the nature of the medium, by the addition of specific chemical reagents, by age, by irradiation, and by various other factors. Most species of bacteria have been observed to show some manifestation or other of the phenomenon. Sometimes the variant forms revert easily to the parent type: sometimes they are stable. The reversion is sometimes sudden, erratic, and apparently spontaneous. Some variants appear to be quite stable, but can be made to revert when subjected to a special treatment. Hadley suggested that rough colonies only reverted when the variant strain had not been thoroughly isolated and purified. Yet even stable rough strains have been made to revert by drastic means such as passage through an animal. The different colony forms are linked in varying degrees with other bacterial properties such as morphology, pathogenicity, and biochemical reactions.

Some observers have thought that the different forms arising by 'dissociation' were parts of a regular bacterial life cycle—just as one might encounter frog-spawn, tadpoles, and frogs in the course of a series of observations. But there is really no evidence for such an idea, which is not generally believed in.‡

There will be no need to discuss the many observations on colony form in detail.§ Qualitatively they conform to the pattern of other

† P. Hadley, *J. Inf. Dis.*, 1937, **60**, 129; K. Roelcke and H. Intlekofer, *Z. Bakt.*, 1938, **142**, 42.

‡ I. M. Lewis, *J. Bact.*, 1937, **34**, 191; L. F. Rettger and H. B. Gillespie, ibid., 1933, **26**, 289; 1935, **30**, 213.

§ The following are some typical studies and discussions: P. Hadley, loc. cit.; F. Faragó, *Z. Bakt.*, 1934, **133**, 139; R. T. Scholtens, ibid., 1937, **139**, 467; C. S. Flynn and L. F. Rettger, *J. Bact.*, 1934, **28**, 1; E. W. Todd, *Brit. J. Exp. Path.*, 1928, **9**, 1; G. H. Eagles, ibid., 1928, **9**, 330; D. C. B. Duff, *J. Bact.*, 1937, **34**, 49; G. P. Youmans and E. Delves, ibid., 1942, **44**, 127; T. J. Mackie, *Brit. J. Exp. Path.*, 1920, **1**, 213; C. G. Pope and S. Pinfield, ibid., 1932, **13**, 60; S. Spicer, *J. Bact.*, 1933, **26**, 505; G. M. Mackenzie, H. Fitzgerald, and V. Irons, ibid., 1935, **29**, 583; M. D. Eaton, ibid., 1935, **30**, 119; H. E. Morton, ibid., 1940, **40**, 755; E. H. Rennebaum, ibid., 1935, **30**, 625; M. L. Cowan, *Brit. J. Exp. Path.*, 1922, **3**, 187, and many others.

phenomena of adaptation and variation. The production of the variants occurs according to the same scheme as that which appears in the work of Lewis, Bunting, and others referred to in § 2: while the relations of stability and reversion can be seen to form part of the general picture which emerges from the study of drug-adapted or medium-adapted cells.†

4. Environmental influences on variation

To obtain a balanced view of the phenomenon of variation as described in bacteriological literature one should also take into account the opinions expressed by several authors who have laid rather greater stress on environmental influences.

Rettger and Gillespie,‡ having studied morphological changes in *B. megatherium*, reached the conclusion that crowding of cells during growth was a potent cause of variation. They thought that the variant forms only arose when unfavourable influences nearly stopped growth, which, however, could still continue slowly in the face of difficulties. In a later study§ the same authors found that variant cells only occurred after growth and reproduction had nearly ceased. Reimann|| concluded that the frequency of variations in *M. tetragenus* was increased under adverse conditions such as faulty nutrition. He states that ageing for very long periods is the simplest method of inducing type variations. The correlation between the appearance of variants and the inhibition of normal growth is particularly stressed by Haddow,¶ who studied the production of variant colonies with the object of finding whether this phenomenon would provide analogies which would prove suggestive in the study of the cancer problem.

The last members of a colony to grow will presumably be produced under very abnormal conditions: thus their actual cell substance may well differ markedly from that of the main bulk of the population. It would not be wholly unreasonable to suppose that the lag of such cells in utilizing a new substrate, such as lactose, might be smaller than that of the normal cells. They would then appear to be specific variants. The question arises whether variants in this sense would make their appearance as a constant fraction of the total

† VII. 9. ‡ L. F. Rettger and H. B. Gillespie, *J. Bact.*, 1935, **30**, 213.
§ H. B. Gillespie and L. F. Rettger, ibid., 1939, **38**, 41.
|| H. A. Reimann, ibid., 1937, **33**, 499.
¶ A. Haddow, *Acta Internat. Union against Cancer*, 1937, **2**, 376.

population (as in the experiments of Lewis and of Bunting). This would be quite possible. The fraction in a colony of individuals which would have grown while, for example, the pH passed through the last tenth of a unit where growth was still possible, would be approximately constant.

Once again we are presented with a certain doubt whether the spontaneous appearance of, for example, a lactose-fermenting variant in the absence of lactose is definitely proved. If there were a really sharp segregation of cells grown under quite constant conditions into two classes, those which would grow easily in lactose and those which would not grow at all, then there would be no doubt about the validity of the idea that spontaneous variants arise. If there were merely a statistical spread in the times required for germination of the different individuals, then the significance of the idea would be less (though it would still be quite in order to regard those cells which multiplied first in one sense as favoured variants). If, however, it is only those few cells which have been grown under quite special conditions of pH, malnutrition, or toxic influence, which show a definite difference in growth lag when transferred to their new medium, then the utility of the idea becomes less still. Everything depends upon the actual range of properties shown by the population when tested for the variant character. This is a matter for further quantitative investigation.

In attempting some sort of synthesis of the various observations we shall, however, accept the idea that, sometimes at least, well-characterized variants may in fact arise in a cell division even when there are no special environmental causes.

5. Theoretical discussion

As we have seen,† there are several types of adaptive response to a changed environment, though these are not necessarily fundamentally different. In one there is a steady increase in growth rate to an optimum on repeated subculture; in another there is a very long initial lag on the first transfer to the new medium; and in a third there seems to be a replacement of one growth mode by another. These are observed in culture experiments with liquid media. In experiments with solid media a somewhat different kind of observation is made: cells from colonies grown on the original medium

† VII. 2.

sometimes appear to contain a small proportion, and not more than a small proportion, capable of growing under a given set of changed conditions. This latter phenomenon is closely related to the second type of behaviour referred to above in connexion with the liquid media.

The interpretation of the facts may start from one of two ends. On the one hand, we may regard the adaptive changes as inseparably linked with growth, and may consider the alteration in the amounts or in the qualitative texture of enzymatic substance which must occur during culture under changed conditions. A coherent account of observations on growth in liquid media can be given in this way. On the other hand, it may be postulated that certain modes of cell division give rise to forms inherently capable of using the new kind of substrate, and that these merely develop when the inoculum is transferred to the new medium.

The question at once arises how such new forms might arise during division. The answer can only be indicated in very general terms. The work of Robinow and others (see p. 226) shows that parts of the bacterial substance constitute a sort of nucleus which is itself capable of dividing. This part of the cell must of course consist of macromolecular substances with highly characteristic configurations and packings and foldings of chains. It also seems quite clear that the specific structure of any one part of the cell must stand in a very close relation to that of adjacent parts. Although there is no particular evidence that bacteria have nuclei playing the same dominant role as those of plant and animal cells, nevertheless one can well believe that some central part of the structure has a very considerable influence upon the configuration of all the rest of the matter in the cell Or again, certain specialized regions may play a part analogous to that played by chromosomes in other cells. At all events, there is little difficulty in accepting the idea that certain localities in the cell may have a determinative effect on all the structures around them. At a certain stage these key structures divide. Now the moment of division seems to depend upon conjunctions of probabilities, similar perhaps to those which determine the moment of a phase change in a simple inorganic system. (For example, supercooled water does not freeze at once, but only after a variable time interval, determined by considerations of probability.) Thus the new structures formed will not always be of precisely the same size, and

consequently their influence on their environment (determined partly by spatial or mechanical factors) will vary to some extent. Thus a certain statistical fluctuation in cell properties is to be expected.† We shall refer to this as the statistical effect simply. Whether it is of significance in adaptive phenomena remains to be seen.

More radical than the statistical effect is the possibility of alternative modes of division of nuclear material.‡ A chemical reaction not infrequently takes a different course according to the way in which a given molecule splits: thus to quote two well-known examples,

$$HCOOH = H_2O + CO \quad \text{or} \quad H_2 + CO_2$$
$$CH_3CH_2OH = C_2H_4 + H_2O \quad \text{or} \quad CH_3CHO + H_2.$$

The relative proportions of the products are determined by the relevant energies and entropies. When the time comes for cell material to divide, the more normal mode would obviously be schematized as follows:

A.A.B.B.C.C.D.D
A.A.B.B.C.C.D.D
becomes
A.A.B.B.C.C.D.D
and
A.A.B.B.C.C.D.D:

but there may be a small chance that

A.A.B.B.C.C.D.D
A.A.B.B.C.C.D.D
gives
A.A.B.B
A.A.B.B
and
C.C.D.D
C.C.D.D.

The diagram represents an extreme case where two cells are produced which presumably would possess completely different characters. But the process indicated might be confined to very restricted regions of the bacterial substance and might then be responsible for the divergence of character in quite small but specific ways. Simple molecules, atomic nuclei, and, of course, most macroscopic objects are all capable of being put apart in alternative ways, and there seems no reason in principle why the same should not apply to various key parts of the macromolecular architecture of the cell.

The modes of division of cell material must be governed by energy and entropy relationships, which are the same in principle as those governing chemical reactions, phase changes, or changes in state of aggregation.

If there is an inherent likelihood that alternative forms of molecular texture may arise in the cell at the moment of division, it is none the less probable—indeed it may be taken as certain—that the

† See Chapter X.

‡ Cf. G. B. Reed, *J. Bact.*, 1933, **25**, 545.

proportions of the different types will vary during growth, and that the types themselves will be subject to certain qualitative modifications when presented with new building material slightly diverging from that which has hitherto been supplied. From this point of view the question would no longer be: what is the mechanism of variation and adaptation, but, which of the various mechanisms plays the more important part in any given example.

Something can be said about this on grounds of general probability. In the first place, it seems dangerous to invoke too frequently the radical modification of basic pattern which could be brought about by abnormal division. This would lead to an exaggerated view of bacterial variability. Seeing that a given bacterium can be trained successively to give optimum growth in a whole series of carbohydrates, and that training to a series of drugs can be superposed upon this first set of adaptations, one can only deny the active participation of the training agent at the cost of assuming an almost unbelievable liveliness in the spontaneous variability. But in ordinary circumstances the main species characteristics of bacteria remain fairly stable,† and phenomena like the transformation of coliform organisms into streptococci are not observed. It would seem reasonable to suppose that abnormal division requires rather a high activation energy, on the one hand, or a considerable lowering of entropy on the other. The ordinary way of facilitating processes which require high activation energy is to raise the temperature. This is ruled out with living cells since the increased temperature would destroy the protein structures. A special way is to provide local concentrations of energy by means of quanta of radiation. X-rays and ultra-violet light are in fact effective in calling forth mutations in living cells. The quanta can bring about profound rearrangements of chemical bonds in parts of the cell without raising the temperature of the whole. For the favourable entropy fluctuation there is nothing to do but wait, and some species-transformations might occur in this way in the course of nature's unhurried processes in evolutionary ages.

What is a stage more likely than the production of fundamentally new patterns by abnormal division is the formation of a certain proportion of cells containing a specially large supply of some existing pattern. Suppose this is one by which a new substrate such as

† Cf. A. I. Virtanen, *J. Bact.*, 1934, **28**, 447.

lactose is used, then these cells will be the first to grow, and, if the lag for the new process is long and the conditions are difficult, they may be the only ones capable of doing so. In this way the *Bact. coli mutabile* kind of phenomenon could arise. (In this connexion the assumption of a long lag attending the utilization of the new substrate is not arbitrary or gratuitous, since we know that normal cells in their normal habitat always develop a lag when active reproduction has been suspended.) There should be a whole range of possibilities, extending from that where nearly all the parent cells succeed in growing in the new medium to that where only very exceptional ones survive at all. Where we meet with little lag in the new medium, and observe simply a progressive training in respect of actual growth rate, it is pretty clear that nearly all the cells in the inoculum take part in the adaptation. Where, on the other hand, there is a very long initial lag, the interpretation is ambiguous: the lag of all the cells might be long, or the apparent lag might arise from the very small fraction of the inoculum which in fact is able to grow at all. Probably the correct interpretation varies from case to case.

Interesting considerations arise in connexion with the stability of adapted or variant forms. At first sight, the fact that the variants are sometimes stable towards reversion is most naturally explained by the idea that they have arisen from abnormal cell divisions, and that the latter have created fundamentally new patterns which, thereafter, are reproduced indefinitely. Moreover, according to this view, a real discontinuity is involved, since a mere statistical fluctuation in the amount of some cell pattern would not be inherited. On closer inspection, however, the matter assumes a different complexion. In the first place, stability is rarely, if ever, absolute. Both Massini himself and Baerthlein have reported reversions of the *Bact. coli mutabile* variants usually regarded as stable:† stably sulphonamide-trained *Bact. lactis aerogenes* reverts on growth in presence of proflavine;‡ proflavine-resistant organisms of the same species which are stable to dozens of passages in ordinary synthetic media revert in presence of phenols;§ stable *R*-variants of certain bacteria revert on passage through animals. It seems that the state is one of metastability rather than of true stability. Thus the discontinuous mutation view would seem to predict too great a degree of stability for the majority of adaptive changes. In the second place, one can only

† p. 171. ‡ p. 124. § p. 117.

test whether, for example, a lactose-utilizing variant has been formed by allowing growth to occur in presence of lactose, during which process there is opportunity for stabilization to occur in other ways, as already discussed in the last chapter.

Another argument based upon the phenomena of reversion operates in a direction which is the opposite of what might be expected at first. As has been explained, adapted cells sometimes pass through a sequence of stages, from which they show first, very easy reversion, then difficult or delayed reversion, and finally achieve relative stability (or metastability).† It might be supposed that the following would give the simplest explanation. Initially a discontinuous production of variants occurs: on repeated culture in the 'training' medium, the purity of the variant strain becomes greater and greater as the original type is eliminated. When the variant strain is impure, that is, in the early stages, there is easy reversion due to re-emergence of the original strain on return to normal conditions; when the variant strain has been completely freed from the original type, no reversion is possible any longer. Stabilization, on this view, is simply the purification of the variant type. It will be shown in the chapter on selection‡—and the argument need not be anticipated here—that the reversion (in some examples at least) *cannot* in fact be attributed to a shift in the balance of population between adapted and unadapted types. Stabilization, therefore, is a process affecting the contents of individual cells. Since it is conditioned by long-continued growth in the appropriate environment, it is not reasonable to refer it to an initial discontinuous change at one particular cell division.

The general conclusion of the discussion of this section is that while the production of abnormal configurations at the time of division is likely enough in principle under special circumstances, and while some phenomena of variation or adaptation may well be governed by this mechanism, changes in cell material, both qualitative and quantitative, occurring during actual growth, will probably play an even more important part. Further discussion of the question is reserved for the chapter on selection.

6. Influence of radiation on bacteria§

The most obvious effect of ultra-violet light or of X-rays on bacteria is to kill them. The number of survivors declines exponentially

† See pp. 112, 163, 166, and 185. ‡ IX. 3.

§ D. E. Lea, *Actions of Radiations on Living Cells*, Cambridge, 1946.

with time.† But in those cells which survive, mutations may occur.

For example, Lincoln and Gower‡ exposed *Phytomonas stewartia* to X-rays of such intensity that only about one cell in 10^5 survived. Mutations were observed as indicated by change in the size and appearance of the colonies formed on plating out. The authors remark that the mutations differed only in frequency from those occurring spontaneously. They lay stress on the discontinuous nature of the changes which take place, and conclude that bacterial characters are localized in definite 'genes'.

Gray and Tatum§ obtained two mutants from *Bact. coli* by exposure to X-rays. One required biotin for growth, the other threonine. In an analogous way *Acetobacter melanogenum* gave four mutants specifically requiring glycine, glycine or *l*-serine, adenine or adenosine, and leucine, respectively.

In view of the potentially lethal action of the radiation it is not surprising that specific parts of the structure of the bacterial substance should be destroyed. What is interesting is that it cannot be regenerated. This shows that there is considerable localization of the specific characters.

7. Differences between bacterial species and genera

As an appendix to the consideration of variation and adaptation it is of interest to inquire into the degree of fixity of bacterial types and about the possibility of their interconversion.

The classification into the main species is fairly well defined, but within a given species it often seems that a continuous series of intermediate forms may exist.‖ Numerous studies have been made of the coliform group,¶ in which all observers seem to be agreed that innumerable fine gradations of properties occur. To mention only one example, the behaviour towards lactose varies almost continuously. One of the main sub-divisions of the species is into the

† D. E. Lea, R. B. Haines, and C. A. Coulson, *Proc. Roy. Soc.*, 1936, B, **120**, 47; 1937, **123**, 1; J. A. Crowther, ibid., 1926, B, **100**, 390.

‡ R. A. Lincoln and J. W. Gower, *Genetics*, 1942, **27**, 441.

§ C. H. Gray and E. L. Tatum, *Proc. Nat. Acad. Sci.*, 1944, **30**, 404.

‖ Cf. e.g. P. Fildes, *Brit. J. Exp. Path.*, 1927, **8**, 219.

¶ L. W. Parr, *Bact. Rev.*, 1939, **3**, 1; C. Nyberg, K. Bonsdorff, and K. Kauppi, *Z. Bakt.*, 1937, **139**, 13; O. Sievers, ibid., 1937, **139**, 27; R. P. Tittsler and L. A. Sandholzer, *J. Bact.*, 1935, **29**, 349; I. E. Minkewitsch, D. J. Rabinowitsch, and F. S. Joffe, *Z. Bakt.*, 1936, **137**, 152.

lactose fermenters and the lactose non-fermenters, the former including *Bact. coli* and *Bact. lactis aerogenes* (sometimes called *Escherichia* and *Aerobacter* respectively) and the latter including important pathogenic organisms such as *Bact. typhosum*. Yet among the lactose fermenters there is *Bact. coli mutabile* which only acts after a very prolonged lag, and *Bact. lactis aerogenes* which needs appreciable initial adaptation to utilize lactose, the original activity towards the sugar being quantitatively a quite variable character. A rather important sub-division is that between the *Bact. coli* strains which are predominantly of faecal origin (*Escherichia*) and strains of *Bact. lactis aerogenes* (*Aerobacter*) which are largely derived from the soil. A population of the *Escherichia* type placed in the soil appears to be succeeded by one predominantly of the *Aerobacter* type, and it is at least possible that the two types can change one into the other. Nyberg, Bonsdorff, and Kauppi have recorded observations on changes from *Escherichia* to *Aerobacter* in isolated strains, and there are at least suspicions of other transformations.

Unfortunately this is one of the subjects where the faultiest technique may lead to the appearance of the most spectacular results, so that great reserve is always exercised in the face of apparent changes. The practically continuous nature of the spectrum of types is, however, not open to doubt, and is at any rate strongly suggestive of interconvertibility which, within defined limits, is not very difficult. Of more profound changes there seems to be no real evidence.†

† For the part which radiations and cosmic rays might play in initiating changes see Schrödinger, *What is Life?* 1944; O. L. Reiser, *J. Heredity*, 1937, **28**, 367.

IX

SELECTION

1. General

SUPPOSE in a population of individual cells there are n_A of type A and n_B of type B. In a growth medium where both types grow equally well the ratio of n_A to n_B will remain constant. If, however, a sample of the population is transferred to a medium in which the growth rate k_A for type A is greater than the growth rate k_B for type B, then the ratio n_B/n_A will diminish progressively towards zero. If n_B happened to be much greater than n_A at the time of transfer, there would clearly be a complete change in the character of the population on continued growth in the new medium. This would constitute a simple example of selection, and one can see quite easily that differential death-rates would lead to the same sort of result. One hypothesis, which has not been without its supporters, attributes most, if not all, adaptive phenomena in bacteria to the operation of selection. The part played by selection must now be considered.

It might be thought strange that so obvious an explanation of the response to changed environment has not been discussed in detail at an earlier stage. The reason for deferring lies in the nature of the arguments which have to be used. With suitable auxiliary assumptions some form of the selection hypothesis can be made to account for nearly all the facts: but it is because these auxiliary assumptions themselves appear to increase in arbitrariness and complexity as one proceeds, that one concludes by declining the main thesis as improbable. To decline one explanation on grounds of probability is, however, difficult unless an alternative has been prepared. In the light of the previous discussions, we are now in some sort of position to decide whether the alternative is one to which preference can in fact be given. Moreover, to anticipate the conclusion which will be reached in the present chapter, although selection is probably not the primary mechanism of bacterial adaptation, it is one which must be superimposed upon other adaptive processes. Hence the discussion of it comes more appropriately after that of those primary changes which its operation may accelerate or intensify.

The ways in which bacteria could develop immunity to drugs or

acquire a capacity for optimum growth in a new medium might be classified as follows:

(1) Selection of a type favoured by the new environment and pre-existing in the original population, assumed to be heterogeneous.

(2) The spontaneous formation of variant cells at the time of division and the preferential growth of those which happen to be favourably endowed.

(3) The direct action of the new environment in causing (*a*) the operation of alternative modes of growth, (*b*) the quantitative increase of certain parts of the cell material, (*c*) the qualitative modification in the texture and configuration of certain parts of the cell material, (*d*) a mode of cell division likely to favour growth in the new environment itself.

The hypothesis that we are normally concerned with a selection of pre-existing strains from a mixed population is very improbable indeed. In the first place, variant strains have frequently been observed to arise from cultures which sprang originally from a single cell.† Thus at some stage the parent type must have become heterogeneous, and the modification of the individual cell has to be accepted. This argument seems to be absolute in logic and can only be met by challenging the observations, which, however, are rather numerous. The second argument is one of probability. Adaptation can easily result in the acquisition of a very considerable number of new characters, which may not only be held in various combinations, but may be developed independently and at different rates. As an example of the multiple combinations one might cite the strains of *Bact. lactis aerogenes* adapted to various combinations of sugars (p. 167). The different rates at which different characters appear on adaptation is exemplified by the work of Reed on *Serratia marcescens*‡ and by observations on a slow-growing strain of *Bact. lactis aerogenes* during training to utilize a synthetic medium.§ To account for the facts, it would be necessary to postulate the pre-existence of an almost unlimited series of sub-strains. Since the number and variety of these would increase with every new adaptive experiment performed, one would soon arrive at something like a *reductio ad absurdum*.

† e.g. J. C. Torrey and E. Montu, *J. Bact.*, 1936, **32**, 329; K. H. Dombrowsky, *Z. Bakt.*, 1936, **137**, 160.

‡ G. B. Reed, *J. Bact.*, 1937, **34**, 255.

§ R. M. Lodge and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1943, **39**, 420.

It seems clear then that if selection is to operate, the strains must be interconvertible. As we saw in the discussion in the last chapter, spontaneous variations are possible in principle, and many bacteriologists have emphasized the manifold potentialities of the cell.†

But there are many improbabilities about the idea that spontaneous variations can be the source of most adaptive changes. Adaptation is so nicely adjusted to the conditions of training that one has great difficulty in supposing the initiating mechanism to be a random change. When bacteria are trained to resist drugs the degree of adaptation is exactly graded to correspond to the concentration of drug in which the cells were grown (Chapter VI). This quantitatively adjusted response is much more easily and naturally explained by a shift in the enzyme balance of all the cells than by selection of a few which happened to be formed with an accidental immunity. If the degree of acquired immunity bore no particular relation to the training concentration in general, then one could the more easily imagine it to have arisen by a spontaneous variation. Even so, with arbitrary auxiliary hypothesis the combination of spontaneous variation with selection could be made to account for the facts, as will be discussed in a later section.

Another rather weighty objection to any form of pure selection hypothesis is provided by the facts about reversion. Drug-trained cells will often lose their adaptation when serially sub-cultured in absence of drug. At the same time it can be shown that the growth rate, in the drug-free medium, is the same for the immunized cells as for the original untrained cells. There is thus no reason why selection should help the latter to regain their numerical ascendancy. The adaptation, however, is lost, and the natural conclusion is that it has been lost by changes in the individual cells themselves. Again, training can in certain examples be carried to a length where no reversion occurs even after very many sub-cultures in the original environment. According to the selection hypothesis the stabilization would occur at the stage where all members of the original unadapted strain had been eliminated. Yet one of these stably adapted strains, *ex hypothesi* now a pure strain, can suffer induced loss of adaptation under appropriate conditions (pp. 117, 124). This reversion

† Cf. the discussion by R. R. Mellon (*J. Bact.*, 1942, **44**, 1) of what he calls the 'polyphasic potencies' of the bacterial cell.

must depend upon changes in the individual cells. The evidence on this matter is considered further in § 3.

Hypotheses about the direct influence of the environment on the actual growth of the cell have been discussed in previous chapters and need not be further dealt with here, except that what was classified above as 3 (*d*) calls for some comment. What is envisaged here is an interplay of the changing material balance in the cell and the actual mode of division. Suppose there is some localized matter in the cell with functions akin to those of a nucleus or centre of organization. The composition and texture of this might change as it expanded to the point of division in presence of a new substrate, or in presence of a drug. As a direct result of this change the mode of division might be modified so as to give a variant type of cell. Since, however, the modification of the supposed nuclear material occurs during its own expansion in the altered environment, the mechanism is really very little different from one which might operate on the cell as a whole.

The next few sections will be devoted to a brief review of certain specific phenomena from the two points of view of selection and of direct adaptation.

2. Training and reversion

As we have seen in a good many examples, training, whether to drugs or to new substrates, is progressive through a series of sub-cultures. In general, reversion occurs easily if the cells are returned to the original environment after a short training: after longer training there is delayed reversion; and eventually something like complete stability is reached. *With suitable assumptions* the selection hypothesis will account excellently for this pattern of events.

Suppose we have two types of cell A and B present in numbers n_A and n_B. The B type may be thrown off in small proportion as variants during growth in medium I, but does not grow itself so well as the A type. Let the growth-rate constants be k_A and k_B respectively. In this medium I, the numbers settle down to a definite ratio such that n_B/n_A is small. Now let an inoculum be transferred to medium II in which the growth rate K_B of B is greater than that, K_A, of A. We shall have, after time t,

$$n_A = (n_A)_0 e^{K_A t}$$

and

$$n_B = (n_B)_0 e^{K_B t},$$

whence $$n_A/n_B = \frac{(n_A)_0}{(n_B)_0} e^{-(K_B - K_A)t}.$$

Since K_B is greater than K_A, n_B will increase indefinitely at the expense of n_A. As soon as the ratio is preponderatingly in favour of the B type the culture will appear to be adapted. n_A diminishes steadily. Now although the equations written down refer to continuous variables, cells are individual countable entities, and as soon as n_A corresponds to less than one cell in the inoculum transferred in a serial sub-culture, there will be none of the A type left at all. If, then, the strain consisting entirely of type B is returned to the original medium I, there will be no cells of type A to increase in number, in spite of the fact that k_A is much greater than k_B. The bacteria remain now a homogeneous population of type B and at any subsequent re-transfer to medium II will show optimum growth. That is to say, the training will appear to be stable. If K_B is considerably greater than K_A, the culture in medium II will appear to be practically fully trained as soon as n_B has increased to be even comparable in magnitude with n_A. For example, if n_B constituted one-half the total number of cells, and if only the B type grew at all, the difference between the mixture and a pure culture of type B would only reveal itself by a lag in medium II corresponding to the time taken for the 50 per cent. of B in the inoculum to double itself, that is to say, to one generation time. This would be quite a small apparent lag. But with such a mixture the strain A would be able rapidly to regain the upper hand when returned to medium I, that is to say, there would be rapid reversion. The phenomenon of delayed reversion would be met at the stage where, in medium II, n_A had fallen almost but not quite to zero. We suppose that an inoculum consisting of a very few cells $(n_A)_0$ of type A and a large number $(n_B)_0$ of type B is returned to medium I. After time t, the numbers will be

$$n_A = (n_A)_0 e^{k_A t} \quad \text{and} \quad n_B = (n_B)_0 e^{k_B t},$$

respectively. If $(n_A)_0$ is small enough it will be a very long time before n_A once again outweighs n_B even though k_A is much greater than k_B. During this time n_B will have become very great, that is to say, there will have been a large number of sub-cultures in medium I. Not until n_B has fallen very considerably behind n_A will any loss of power to grow in medium II become apparent. Hence the delay in the detection

of reversion. The delay is, of course, only apparent, since the readjustment is in progress all the time, but the nature of the exponential growth law is such that only when there is a very serious departure from 100 per cent. of the strain *B* is its decline detectable by growth tests in medium II.

If, in the original culture in medium I, there were an extremely minute proportion only of type *B*, and if, further, type *B* alone were capable of growth in medium II, then on transfer for the first time, there would be a very long apparent lag. In the ordinary way a lag is calculated on the assumption that all the cells grow. If the inoculum consists of $(n_A)_0+(n_B)_0$, and if only $(n_B)_0$ actually grow, then, over and above any real lag, there will be an apparent lag equal to the time required for $(n_B)_0$ to increase to $(n_A)_0+(n_B)_0$. If the former is a minute fraction only of the latter, this lag will be long. Furthermore, if only the *B* type grow in medium II, the resulting strain will be nearly pure *B* and reversion will be difficult or impossible. The long initial lag on transfer to the new medium, and the stability of the strain which eventually grows, are just what is found in such examples as the adaptation of *Bact. coli mutabile* to lactose† or of *Bact. lactis aerogenes* to *d*-arabinose.‡

It is evident from what has been said that many of the experimental facts are very well accounted for by the selection hypothesis. The alternative, to recapitulate the discussion of Chapter VII, is to suppose that changes in the enzymes occur during training, and that the new patterns or configurations only become stable when the original ones, which might act as nuclei, in the crystallographic sense, for reversion, are eliminated; or, alternatively, that the adaptation only becomes stable when the strains set up by enzyme expansions have been eased by the complete renewal of all the cell material many times over.

In spite of all their superficial differences, the two major points of view—selection of certain members of the population, on the one hand, or replacement of old enzyme material by new all through the bacterial mass, on the other hand—have in the last analysis much in common. Taking the bacterial mass as a whole, each postulates the rise to predominance of a new kind of structure during training: each supposes this new type of structure only to be stable when the renewal is complete. The selection hypothesis makes the cell itself

† p. 168. ‡ p. 169.

the unit, and focuses attention upon changing proportions of cells of different types. The hypothesis of direct adaptation makes the change occur in all cells, and focuses attention upon the changing proportions of intracellular components. Since in most experiments we deal with total bacterial mass, it is seen not to be surprising that the two hypotheses so often lead to the same formal results. This means that the distinction between the two by experimental tests becomes more difficult, but it also means in one way that the distinction between them becomes of slightly less importance.

To take the example of *Bact. coli mutabile* and lactose: according to the one view, there is a long initial lag because only very few of the original cells are capable of utilizing the sugar; according to the other, it is because in a given cell there is only a minute amount of enzyme capable of doing so. Integrated over the bacterial mass, there is, according to either view, only a small amount of substance capable of utilizing lactose, and on growth in presence of the sugar this increases. The distinction between the two views is reminiscent of the contrast between two possibilities, each often enough met with in practice, namely, one where a chemical change proceeds more or less uniformly throughout a volume of substance, and the other where it spreads from a few localized nuclei.

Looked at in this way, one is inclined to feel that a complete gradation of behaviour is probably to be detected, by the proper methods, among bacteria, just as it is known to exist in ordinary chemical reactions, where the relative importance of nucleation and growth of nuclei varies widely. Nevertheless, it remains true that direct modification of the enzyme balance or texture by growth in the changed environment provides a more natural way in which the changes can be initiated. Nor does one see why such changes should, *in general*, be initiated in some cells very much more easily than in others of the same type, though a certain statistical variation is to be expected.

3. Direct tests of changing population balance

The most serious objection to the form of selection hypothesis outlined in the last section is that, in certain examples at least, the relations which have to be postulated between the values of k_A, k_B, K_A, and K_B do not correspond to reality. Shortly, if a strain is to revert when returned to the original medium, then, according to the

selection hypothesis, this can only be because the trained cells are at a relative disadvantage in it (that is k_B is less than k_A). This appears sometimes to be definitely not so.

Bact. lactis aerogenes after being cultured repeatedly in a glucose medium, may be transferred for training to a corresponding medium with glycerol instead of glucose. In the early stages of adaptation to glycerol it reverts if returned to glucose: yet the growth rate of glycerol-adapted cells is no smaller in glucose than that of the unadapted cells. There is thus no reason why a change in the balance of the population should occur.

It might be said that independent tests of the growth rates of the trained and untrained types are not delicate enough to reveal a small differential rate in favour of the untrained type. To test this question, the following experiment was made. Cells were trained in glycerol medium until they became completely stable. They were then mixed with an equal proportion of completely untrained cells, and the composite strain was sub-cultured many times in the glucose medium. If, normally, non-reversion depends upon the absence of any residue of the untrained type, and if reversion is simply due to a more ready growth of the untrained strain, then this composite culture should show a loss of adaptation as rapid as that shown by a partially trained one. No loss in the glycerol adaptation in fact occurred, in spite of the deliberate addition of the considerable proportion of untrained cells.† Thus one must conclude that reversion, where it is observed, is due to some cause other than a shift in the population balance.

In a similar way it has been shown that a mixture of proflavine-adapted cells and of non-adapted cells do not appreciably change their proportions when grown in the absence of proflavine, so that, here, too, the reversions observable in appropriate circumstances must be due to causes affecting individuals rather than the balance of populations.‡ The experiments were made in the following way. A strain was trained to a very high concentration of proflavine, but so as not to be completely stable (see p. 115). On passage through the proflavine-free medium it showed reversion to the 'equilibrium' state previously described. In this state it showed an unchanging form of lag-concentration curve when tested in proflavine at intervals

† Experiments by E. G. Cooke in the author's laboratory (in the press).

‡ Experiments by J. M. G. Pryce in the author's laboratory (in the press).

in a long series of sub-cultures in the ordinary drug-free medium: no further reversion was occurring. This culture was then plated out on agar and about ten colonies selected, which were sub-cultured in the synthetic medium and tested for their proflavine resistance. This was found to be very different for the strains taken from the individual colonies, and almost zero for some. Thus, either the growth on agar had caused some further reversion, or the 'equilibrium' strain had been a mixture of reverted and less reverted types. A second sub-culture of one of the isolated strains gave a series of secondary strains all showing the same proflavine resistance. It seemed, therefore, that the growth on agar was not responsible for the heterogeneity found in the first series of colonies. Thus the 'equilibrium' strain had indeed been a mixture of proflavine-trained and some almost untrained cells. Yet it had preserved the precise form of its lag-concentration relationships with proflavine through many sub-cultures. Thus the conclusion was that such reversions as occurred in other circumstances were not due simply to a changing population balance.

These various results may be summed up briefly by saying that neither with substrate adaptation nor with drug adaptation do mixtures of trained and untrained cells change their proportions on sub-culture in the way which would have to be postulated if the pure selection theory were adopted. Consequently, the individual cell appears to be the seat of the essential adaptive changes.

4. Other formal comparisons of selection and direct adaptation mechanisms

(*a*) *Rate of training.* According to the selection hypothesis the rate of adaptation depends upon the difference between the growth-rate constants of the two strains. This may have any value, and the most diverse rates of training could be accounted for. If the 'unadapted' strain does not grow in the new medium, and if the 'adapted' strain is present initially in very small amount, there will be a long initial lag, as already explained: the length of this will be determined by the proportion of the favoured strain initially present.

According to the hypothesis of direct enzyme modification, the rate of training under optimum conditions should, as was discussed in Chapter V, be very great. In favourable circumstances, adaptation should be sensibly complete as soon as the new bacterial substance

formed by growth in the new medium outweighs the initial amount by any considerable factor. But this maximum, although sometimes observable (see for example p. 113) is not usually reached: indeed the general picture is of a very considerably slower adaptation. This is partly connected with the reversibility of the change in its earlier stages. Sulphonamide adaptation is partially lost when partially trained cells are merely allowed to age, and analogous effects probably play their part in other examples. Another factor contributing to the limitation of adaptation rate is the simultaneous operation of alternative mechanisms (p. 176) which lead to a differential effect similar to that which would occur in a selection process.

The whole subject of adaptation rate is in need of further clarification.

(*b*) *Composite growth curves.* The interpretation of the composite growth curves met with in the study of adaptive processes has been discussed from the point of view of alternative reaction mechanisms in an earlier chapter. The interpretation in terms of selection would be simpler still, and in some respects more satisfactory, were it not for the inherent improbabilities of and other objections to that hypothesis. The two separate components of the growth curve, as illustrated in Figs. 35, 49, 50, and 53 would, if there were separate strains in competition, have a quite independent existence. The greater lag of the faster-growing strain would, however, on this basis, be an apparent one and due simply to its small initial numbers. The relations are illustrated diagrammatically in Fig. 61. According to this interpretation, however, one would expect a culture which had once grown far enough to pass the transition point to be stable to reversion, which is not by any means necessarily found.

(*c*) *Families of lag-concentration curves.* As we have seen, there are good examples of a precise numerical correspondence between the properties of an adapted strain and the concentration of the drug at which training is carried out.† This would certainly not be possible as a result of simple selection from a mixture of a few non-interconvertible types differing among themselves in their sensitivity to the drug. It would only be possible if there existed a continuous spectrum of such strains, and even then a special assumption would have to be made: namely, that of a particular law for the distribution of natural drug resistance among the members of the population.

† Chapter VI.

If one specifies the degree of resistance by the value of the drug concentration, $\bar{P}$, to which a given type of cell is immune, then the frequency distribution of $\bar{P}$ values among the cells would have to be a remarkably steep one. It would be necessary to suppose that, of all the cells with $\bar{P}$ greater than any assigned value $\bar{P}_1$, the vast majority had values very close to $\bar{P}_1$ itself. (This form of statistical distribution is not, of course, unknown. One example familiar to

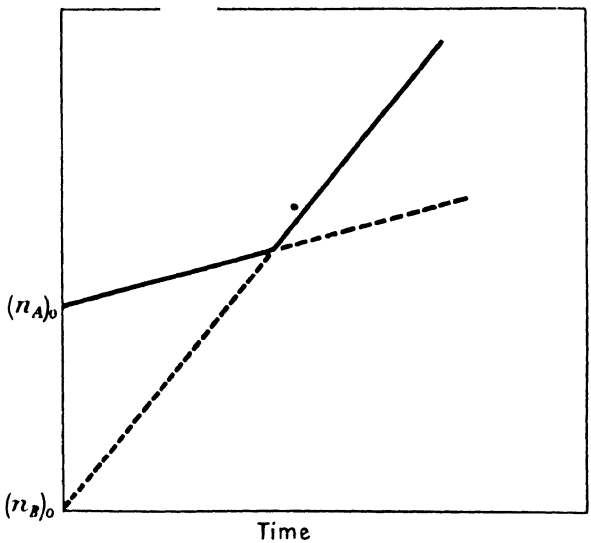


FIG. 61. Composite growth curve on selection hypothesis.

chemists is the distribution of activation energies among molecules. Of all the molecules possessing energies greater than some large amount E, the vast majority have them in a narrow band quite close to E itself.) This form of distribution would ensure that when cells immune to $\bar{P}_1$ had been selected, there would be among them a residue of still more resistant cells for further selection, but too few to give the culture as a whole an effective resistance greater than that corresponding to $\bar{P}_1$ itself.

The difficulty is that while the energy distribution law to which reference was made is a natural consequence of the kinetic theory, the frequency distribution law for the supposed drug resistances is entirely arbitrary. The view that drug resistance develops by direct

modification of the enzyme systems during growth is, on the other hand, susceptible of a simple and rational interpretation.

One respect in which the pure selection hypothesis might appear at first sight to have an advantage is in accounting for the definite 'equilibrium' levels of adaptation at which cultures may settle down.

We may recall that cells can be trained to resist very high concentrations of proflavine, that is, $\bar{P}_1$ is very great. On serial sub-culture in the drug-free medium, there is partial reversion to a stable limit corresponding to $\bar{P}_2$. The value of $\bar{P}_2$ depends upon the length of time for which the training was originally carried on. According to the selection view, one would assume that the cells trained at $\bar{P}_1$ developed a distribution with a sharp maximum in the neighbourhood of $\bar{P}_1$ itself, but including values down to a lower limit $\bar{P}_2$. On sub-culture in the ordinary drug-free medium the less resistant cells would be assumed to have an advantage which allowed them to increase in relation to the others. Thus $\bar{P}$ for the culture as a whole would move down towards $\bar{P}_2$. This would be the lower limit, since cells with smaller values are assumed to have been eliminated entirely. The longer the original training was continued the more closely would $\bar{P}_2$ approach $\bar{P}_1$ and the higher would be the level of the 'equilibrium' strain.

The difficulty about all this is that, as has been explained, when such shifts of population are tested for in artificially prepared mixtures of strains, they do not happen. The real explanation of the reversions to 'equilibrium' levels must be simply that in the trained culture some of the cells have had time to become stably adapted and others not. Some therefore revert and others do not. Moreover, the extent of enzyme expansion which can be tolerated in the cell without instability depends upon the thoroughness with which concomitant adjustments have occurred, as has been discussed on p. 187.

5. Superposition of adaptation and selection

There is no escape from the conclusion that once, by whatever mechanism, certain cells have become better adapted than others to grow and multiply in a given medium, selection must automatically be superimposed on the other adaptive process.

On the one hand, in the course of training, it will accentuate and accelerate the operation of other mechanisms, since the first cells to become adapted, even if they owe their priority only to chance, will

have an opportunity to outgrow the rest. In the limit, it is only necessary theoretically for one cell to have become adapted initially. This adaptation will, however, have been conditioned by the drug concentration, or by the nature of the substrates in the medium, so that the degree of immunity will correspond. Thus the rate only, and not the final extent or the character of the adaptation will be modified by selection.

How far the different cells in a medium will vary in the ease with which they acquire adaptive characters is hard to predict. They will have a certain range of ages, and this will cause a slight spread in their responses to the new environment; they will show a certain distribution of sizes, which will be reflected in slight variations in rate of food supply; their division times exhibit some degree of statistical fluctuation. The summation of many small effects gives rise to what will appear as a random variation. With a carefully grown parent culture, and with well-controlled experimental conditions, one would not expect the total range of variation to be great; and it would be surprising if most of the cells did not respond almost at the same time to the simpler and easier forms of adaptation. Where the adaptive process is long and difficult the range of variation might be expected to be much greater.

As selection will accelerate adaptation, so, conversely, it will greatly retard the loss of adaptation. Suppose a population of cells is subjected to conditions in which the adaptation tends to be lost. The algebraic form of the growth law is such that a very small proportion of survivors of the adapted strain will, in any test, still give the culture as a whole the appearance of being largely adapted. For example, if the reverted cells show a lag of 1,000 minutes in presence of a drug, then 10 per cent. of immune cells, showing no lag, and having a generation time of 30 minutes, will give the culture as a whole an apparent lag of about 100 minutes only.

An approximate quantitative calculation† of the behaviour of a mixed strain containing adapted and reverted cells is of some interest.

Suppose there are, for example, 1 per cent. of immune cells. Let us consider separately the behaviour of the two parts of the population when inoculated into a medium containing the drug to which

† D. S. Davies, C. N. Hinshelwood, and J. M. G. Pryce, *Trans. Faraday Soc.*, 1945, **41**, 778.

the cells were originally adapted. In one case we imagine that we inoculate with 99 sensitive cells, and in the other with 1 immune cell. But in each we reckon the total inoculum, for the purpose of calculating the lag, as 100. We plot two curves showing the relation between the drug concentration and the apparent lag (that is, the value found by extrapolating the growth curve back to 100).

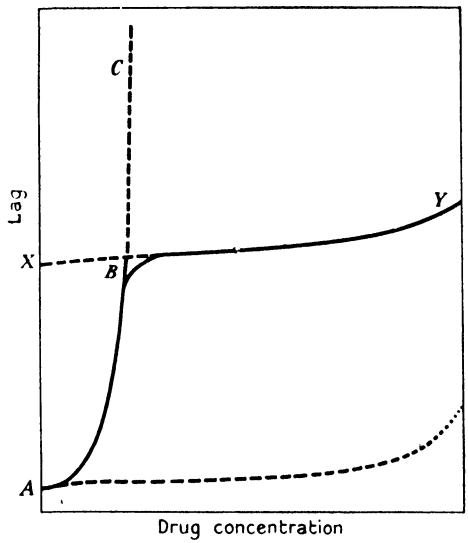


FIG. 62. Lag-concentration curve for mixed strain of immune and non-immune cells.

In Fig. 62 the curve ABC is that for the reverted cells, which constitute the majority. XBY is that for the immune residue. XBY lies well above the true lag-concentration curve for adapted cells because it is calculated on the assumption that the inoculum is 100, whereas the inoculum is in reality only 1. X in fact lies above the real value by the time taken for the number to increase from 1 to 100, whereas A only lies above it by the time taken for the number to increase from 99 to 100.

Now let us consider the actual behaviour of the mixture of 99 sensitive cells and 1 immune cell. The two substrains cannot strictly be considered to grow quite independently and in a simple competition, since growth intermediates put into the medium by the one will be available for the other. Nevertheless we shall obtain a general

idea of the lag-concentration curve of the mixture by following the line *ABY* which represents everywhere the *lower* of the two values given by *ABC* or *XBY* respectively. At the point *B* the immune strain, despite its initial paucity of numbers, now reaches the threshold of visible growth before the more numerous, but more sensitive strain.

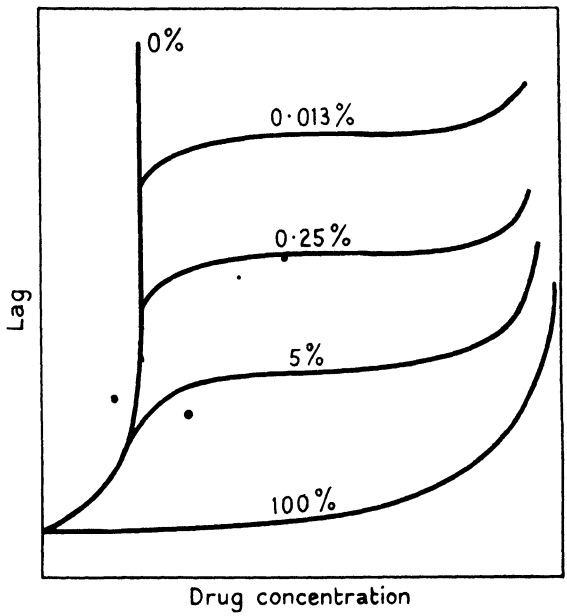


FIG. 63. Lag-concentration curves for various proportions of immune cells (indicated by numbers on the curves).

The type of lag-concentration curve with a shoulder similar to that of *ABY* is not infrequently found with strains of *Bact. lactis aerogenes* which have been trained to high concentrations of proflavine, and which do in fact contain cells with varying degrees of adaptation (see Fig. 31, p. 114).

Fig. 63 shows a family of curves corresponding to various proportions of immune and sensitive cells. As the fraction of immune cells drops, the point corresponding to *B* in Fig. 62 rises higher and higher.

The family of curves in Fig. 63 is in general character not unlike that found for the lag-concentration curves of the *Bact. lactis aerogenes*

strains which have suffered varying degrees of reversion by passage through phenol or cresol after having been trained to proflavine (see Fig. 32, p. 116). Here, then, we seem to have an example of selection of immune cells in the test itself, the result being an overall retardation of the loss of immunity.

It should be emphasized that in the foregoing discussion selection is not regarded as the cause of reversion, which is thought to depend upon factors influencing each individual cell. The selection operates merely in the test for residual immunity, and there makes that immunity appear greater than it would otherwise have done.

X

CELL DIVISION

1. Introduction

As the autosynthetic bacterial substance increases in amount, the surface-volume relations of the cell and the concentration gradients between the different parts of it are modified, and it would be impossible for the normal sequence and balance of processes to be maintained unless the cell divided. To go no further than the primitive model discussed in Chapter IV, it is evident that the ratio of the constants expressing rate of enzyme operation to those expressing rate of loss of intermediates by diffusion must change as the mass of the cell becomes greater. When, however, a steady phase of logarithmic multiplication is established, each cell only fluctuates in size between certain restricted limits: the ratios of constants, therefore, rise and fall to a quite moderate degree about mean values, and it is a justifiable approximation to regard the averages as applicable continuously to the bacterial substance as a whole, as has been done in certain calculations in the foregoing chapters. From another point of view, however, these fluctuations themselves are of superlative importance, since they must be responsible for the periodic division of the cell, upon which the whole possibility of establishing a steady state depends.

In this chapter we shall examine some of the experimental evidence bearing upon the nature of this process.

2. Statistical variation of generation times

By the use of a warm stage one can keep bacteria under continuous observation under the microscope and study the successive divisions of individual cells and their progeny.

Kelly and Rahn† made direct measurements upon very large numbers of cells which were kept growing on agar. Under favourable conditions all the cells continue to divide, there being no 'infant mortality', that is, the throwing off of cells which proved themselves unable to produce fresh daughter cells in their turn. The time between successive fissions was found to be extremely variable. But there was no sort of selection of faster-growing or of slower-growing strains. The progeny of a cell which had divided after an unusually

† G. D. Kelly and O. Rahn, *J. Bact.*, 1932, **23**, 147.

short time showed division times which were, on the whole, neither greater nor less than normal, but simply representative themselves of the general average. The range of variation is illustrated by a result which they quote for *Bacillus cereus* of which one cell did not divide for 185 minutes, the next division following in 25 minutes. On one occasion they observed the second and third divisions to follow one another without any interval at all. Kelly and Rahn suggest that mitosis, that is to say, the internal reorganizations preceding division, may occur without the actual division of the cell into two. This would explain how two successive fissions could occur in very rapid succession.

The distribution of fission times is given in the following table which refers to *Bact. lactis aerogenes* at 30° C., and is taken from among the results in the paper of Kelly and Rahn. The numbers recorded are the numbers of cells out of a total of 323 observed which had fission times within the various 5-minute ranges.

Range minutes	*Number*	*Range minutes*	*Number*
5–10	0	40–45	45
10–15	1	45–50	20
15–20	11	50–55	8
20–25	25	55–60	2
25–30	42	60–65	2
30–35	97	65–70	0
35–40	65	70–75	5

These results have been plotted in the form of a frequency distribution curve in Fig. 64 (with omission of the few examples in the range 70–75 minutes). The numbers have been scaled so that the maximum frequency is represented by the number 100. In the same figure the shaded area is a Gaussian frequency distribution curve so chosen as to have the same maximum and to coincide with the experimental curve at the points where the deviation from the most probable fission time of 35 minutes is equal to ± 5 minutes. The calculated curve is given by

$$y = 100e^{-0{\cdot}0278(x-35)^2}.$$

The experimental curve is evidently not of a strictly Gaussian form, and allows for a considerably greater frequency of large deviations from the most probable value than would be predicted, according to the Gauss equation, from the frequency of the smaller deviations.

Nevertheless, the general shape of the curve suggests strongly that there is some standard value for the division time, and that the

moment of division is advanced or retarded in each individual case by the operation of a multiplicity of factors of a more or less random nature.

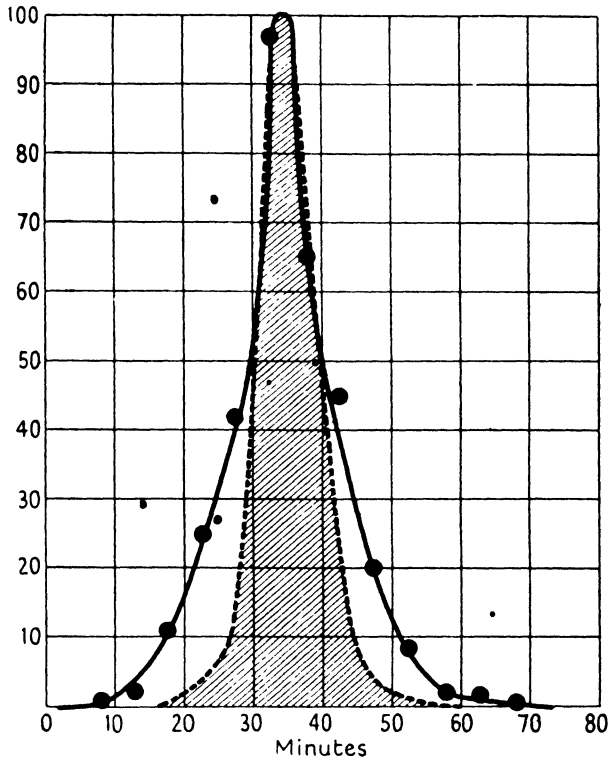


FIG. 64. Distribution of generation times.

One complication, which may affect the precise form of the frequency distribution of the division times, is that there may be two kinds of delay: one in which some important part of the cell material, constituting a sort of nucleus, itself fails to divide; and the second in which, although the internal processes are completed, the formation of the new dividing walls has not taken place.

3. Internal changes preceding division

Appropriate staining methods were stated by Stille† and by Piekarski‡ to reveal specialized regions ('chromatinic bodies') of

† B. Stille, *Arch. Mikrobiol.*, 1937, 8, 125. ‡ G. Piekarski, ibid., 1937, 8, 828.

the cell, which themselves went through regular cycles of division. A detailed study of the multiplication of these structures has been made by Robinow.†

Robinow has worked with *Bact. coli* and with *Proteus vulgaris*. He fixes the cells with osmic acid, treats with warm hydrochloric acid, and stains with Giemsa solution. Special methods of observation are advantageous. With the correct treatment dumb-bell-shaped bodies can be distinguished lying transversely to the long axis of the cell. A young cell may contain only one such body, which, however, presently increases in size and then divides into two closely contiguous dumb-bells. These in their turn increase in width and finally split longitudinally, that is to say, still transversely to the long axis of the bacterium. After the first division of the original dumb-bell body the whole cell may itself split into two by the development of a constriction, but more usually cells possessing a number of these bodies are formed (Fig. 65). These longer bacteria possess a sort of chambered structure, the division of the cell having awaited two or three divisions of the nuclear material, but light transverse partitions being discernible. Robinow observes that 'mono- and multinucleate elements are single and multiple forms of a basic building unit possessing a single chromatinic body'.

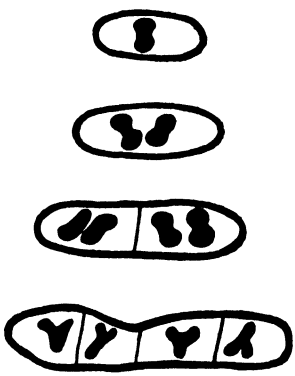


FIG. 65. Division according to Robinow (schematic only).

As to the formation of cell walls, Knaysi‡ states that the deposition of cell wall material precedes the break-up of the cytoplasm in yeasts and follows it in bacteria. In neither is there any indication of division by simple constriction. This agrees in a general way with Robinow, who considers the moment of true cell division to be that when the membranes form between two recently divided chromatinic structures. According to this view most bacteria will really consist of several cells, usually, with young bacteria, two to four.

In certain circumstances (which will be discussed later) bacteria grow to long filaments very many times the normal length. Robinow states that these may sometimes be compartmented, each section

† C. F. Robinow, *J. Hyg.*, 1944, **43**, 413. ‡ G. Knaysi, *J. Bact.*, 1941, **41**, 141.

being complete in itself, and sometimes without any such orderly arrangement and without internal boundaries. Filaments with the chaotic distribution of chromatinic bodies, and without partitions may, he states, arise spontaneously in cultures of *Bact. coli* or *Proteus*, and can also be produced by the action of ionizing radiation in appropriate doses. In such cases it would appear that division, in Robinow's sense, has been inhibited without corresponding inhibition of the growth of cell material. (It seems probable that this latter type of filamentous structure corresponds to that produced under the influence of strongly antibacterial drugs and which will be considered in a later section.)

Robinow remarks that the mode of division which he reveals by his staining experiments 'appears well suited for the transmission of hereditary factors'. He also observes, however, that there is no direct proof that his chromatinic bodies contain nucleic acids, known to be of such profound importance in this connexion with other types of cell.

However this may be—and one might perhaps comment that with non-sexual cell multiplication any part of the cell material would seem to be capable of participating in the transmission of factors, provided that the part in question has the right chemical and configurational character—the really important conclusion to be drawn is that localized happenings inside the cell precede the fission of the whole structure, and may very possibly occasion it.

One thing which this does is to discount the idea that the division of the cell is to be attributed to an effect of surface tension. As is well known, a column of liquid becomes unstable and breaks into smaller masses when the ratio of length to radius exceeds a certain value. The analogy with cell elongation and division is obvious but fallacious. It has been criticized from various angles,† and does not agree with experimental observations. It can certainly not help to explain how the transverse division of the cell as a whole is preceded by the longitudinal division of special parts of its contents.

The basic fact remains that when something—whether it be the whole cell or some definite structure forming part of it—reaches a critical size, then the total configuration becomes unstable and must split into two. What particular manifestation of the universal 'scale-effect' this depends upon remains to be discovered.

† Cf. J. F. Danielli in *Essays on Growth and Form*, Oxford, 1945, p. 305.

In most kinds of cell, other than bacteria, the observable phenomena accompanying division are considerably more complex.† At the moment it remains an open question whether the more elaborate evolutions visible in cells such as those of a fertilized sea-urchin egg constitute merely an embroidery on the simpler theme, and that their understanding will be relatively easy when once the problem of bacterial division has been solved, or whether, on the other hand, there are difficultly observable happenings in the bacterial cell which will only be understood when the fuller and clearer version shown by other cells has been interpreted.

There is no need to describe here the details of mitosis since there will not be occasion to apply them, but one or two general matters should be referred to in passing. In cells showing the typical mitotic or caryokinetic phenomena a certain part of the substance divides into two granules which move apart and constitute two poles between which pass what appear like threads. Other threads radiate in a star-like pattern from the two poles, the whole picture being strongly reminiscent of the lines of force around and between two magnetic poles. Other cell material has formed itself into small more or less rod-like structures which are the chromosomes. These, when the bipolar figure just referred to is formed, move and arrange themselves equatorially and transversely to the bipolar axis. They then separate longitudinally into two (or may have done so earlier), and the divided chromosomes move towards the two poles 'for all the world' as D'Arcy Thompson puts it 'as though they were being pulled asunder by actual threads'. Presently two new nuclei are constituted, a dividing membrane is formed, and the cell divides by constriction. The precise details and the exact order of events vary from one type of cell to another, and the process as a whole is over in from 30 to 60 minutes (Fig. 66).

In view of the existence of crystallizable substances of enormous molecular weight, and of the stretched and unstretched forms of protein chains, one is disposed to believe that the threads and the tensions often referred to in the descriptions of mitotic phenomena may be quite real. Polyfunctional molecules provide a means whereby spatially separated regions of order may be connected mechanically one with the other. Surface tension, osmotic pressure,

† See, for example, D'Arcy Thompson, *On Growth and Form*, Cambridge, 1942, Chapter IV; F. Schrader, *Mitosis*, Columbia U.P., 1944.

and electrical effects are all present. As D'Arcy Thompson† remarks, 'the physical analogies which can be found for various parts of the mitotic process leave me with the impression that when we understand the chemical events in the cell the mechanical evolutions will explain themselves'.

Without entering into the many speculations on the details of the process, therefore, one might try to form a mental picture of cell division somewhat on the following lines.

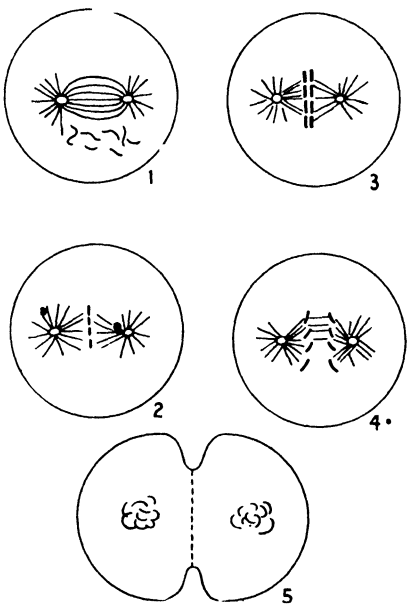


FIG. 66. Some stages in a mitotic division of cell with chromosomes (much simplified).

The cell possesses a certain degree of mechanical heterogeneity, which, however, depends primarily upon chemical differences between regions still preserving a degree of attachment to one another. Certain parts of the structure have a more or less decisive influence in impressing a configuration upon the whole. When autosynthetic processes occur the various parts of the structure increase in mass, and as this occurs the ratio of surface to volume diminishes. Some

† D'Arcy Thompson, loc. cit.

key part of the edifice now becomes unstable and splits into two parts. (The initial instability appears from what has been said to reside not in the structure as a whole, but in some internal section of it, as follows from the mitotic phenomena in cells which show them, and from Robinow's observations with bacteria.)

As to the mechanism of the splitting there is little evidence. Analogies are not lacking: one is the breaking of liquid columns into drops, but this does not seem very helpful. Another is the way in which a large crystal is usually found to be made up from a mosaic of small units, none of which appear during growth to have reached more than a certain size without the development of a discontinuity. This again is too simple. Indications which will be discussed later in this chapter point to the existence of a chemical factor in the process: and it is clear enough in a general way that the chemical balance shifts with the surface–volume relations of the whole cell.

Once the separation of any key structures into two parts has taken place, it will be easy for these to be drawn asunder: any tension existing, for whatever cause, between the two halves of the original and the outer regions of the cell will make this happen. Separations of this kind may go on in a relatively simple way, as apparently with bacteria, or in a more complex way, as with some other kinds of cell. Given, however, the fact that the original total configuration represented, as it presumably did, a maximum of stability for its size, then there must be a tendency for all the contents of the cell now to regroup themselves about the two separated 'nuclei'. This they will do, following the path of least resistance, until the bipolar configuration produced by the initial split becomes as nearly as possible two replicas of the original cell.

The process cannot be completed without the formation of more cell wall, and the question arises as to the mechanism by which this can be regulated according to need. Here again only rather crude generalities can be put forward, but even these are worth while for the sake of outlining a mental picture which later discoveries may fill in.

Since the cell as a whole possesses something of a co-ordinated structure, we may think of a point O, Fig. 67, as the origin of a system of axes. The velocity of any given chemical reaction at the point x, y, z will be a function of the instantaneous concentrations of various substrates and intermediates at that point. These concentrations will themselves be functions of the distances of x, y, z from the surface

of the cell and from the surfaces of the various enzymes which supply the substances in question. The ratios of these various distances, and hence the relative velocities of different reactions, will change as the cell size changes, so that the replacement of other reactions by that leading to wall-forming substance may itself be another result of a scale effect.

But the various possible effects of tension and pressure, of viscosity and of streaming, of diffusion and electrical forces are so manifold that detailed theories must await the key experiment or the decisive theoretical intuition.†

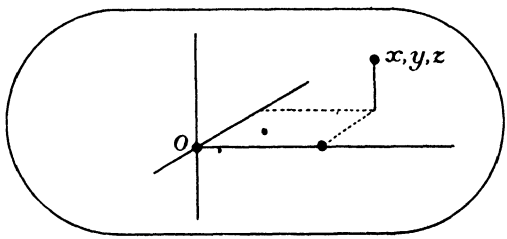


FIG. 67.

In connexion with experiments which seek to reveal the internal structure of bacteria by special staining methods one note of warning must be sounded. The procedures employed are sometimes rather drastic, and not infrequently have the criticism levelled against them that they actually create the structures which they are supposed to demonstrate. One knows that remarkable and varied formations can arise by the coagulation of colloidal systems, so that these criticisms have to be taken seriously, though, on the other hand, it can be answered that standard staining techniques can be calibrated by the use of cells whose internal structure is visible even without the aid of the special treatment.

In some ways the results of investigations with the electron microscope are disappointing, and are subject to the disadvantage that the specimen examined is exposed to very drastic treatment on exposure to the electron beam. Knaysi and Mudd‡ sum up some of their researches by this method in the words: 'The results of the present investigation support the view that different bacteria may

† For a valuable analysis of some possibilities see N. Rashevsky, *Mathematical Biophysics*, Chicago, 1938.

‡ G. Knaysi and S. Mudd, *J. Bact.*, 1943, **45**, 349.

contain nuclear material in different states, and that the state of the nuclear material may change with the development of the cell.' With *Staphylococcus aureus* and *Streptococcus pyogenes* they could detect no internal structure, and concluded that the cells probably contained nuclear material in a state of very fine dispersion. If this conclusion is correct and applicable to bacteria in general, it means that the gap between the bacterial cell and cells showing the typical mitotic phenomena is rather wider than the views of Robinow would suggest. Nevertheless it is not in the least inconsistent with the idea that the initiation of division depends upon an internal process rather than one operating on the cell regarded as a single unit bulk of matter.

4. Cell division and cell morphology

The length attained by a rod-like bacterium depends upon the ratio of the rate of elongation to the rate of division. This is not in general constant throughout the growth cycle. The variations in size were specially studied by Henrici,† who found that, with a few exceptions, such as *Corynebacterium diphtheriae*, the cells were of maximum length in the early stages of the growth cycle, and became appreciably smaller as the stationary phase was approached. In media which support a large total bacterial population the smallness of the cells in a fully grown culture, compared with those formed in the first hour or so after visible growth sets in, is very evident even to casual observation.

Fig. 68 shows some results which are representative. They refer to the growth of *Bact. lactis aerogenes* in an artificial medium. n represents the total count (in arbitrary units, such that the total population finally reached was about 2,000), and ρ is the ratio of the number of cells between 1 and 1·5 arbitrary units long to the number less than 1 unit long. The different curves refer to different concentrations of medium, and to different parent strains. In all cases the proportion of longer cells is seen to fall rapidly as n increases beyond a certain point, which corresponds to roughly half-way towards the end of the logarithmic phase.

5. Delayed division : cells of abnormal length

Although the mean size varies appreciably during the growth cycle, the distribution of sizes at any given moment is, for a normal culture,

† A. T. Henrici, *Proc. Soc. Exp. Biol., N.Y.*, 1921, **19**, 132; 1922, **20**, 179; 1923, **21**, 215, 343, 345.

not a very scattered one, the deviations from the mean being fairly small and, indeed, not much greater than could be accounted for by the different ages of the cells present. This would indicate that the moment of division is not much delayed once the elongation has proceeded to the required extent.

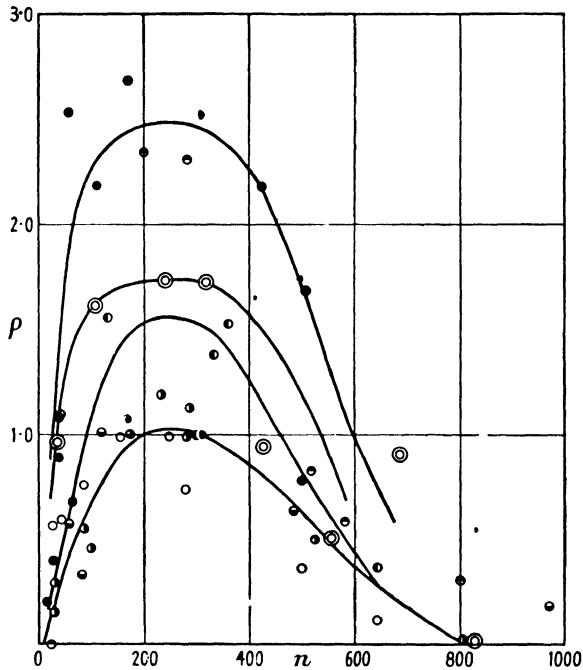


FIG. 68. Variation of ρ during growth cycle for various inocula into glucose media of different concentrations.

In certain circumstances, however, division† may be very considerably delayed, while elongation proceeds, with the result that filamentous or snake-like cells many times the normal length are formed.‡ In extreme cases the failure of division may be so complete that the whole bacterial mass develops as a tangled mass of a few threads, each several hundred times the length of a normal cell.

† Division is here used in the normal sense, not in the special sense proposed by Robinow, see p. 226.

‡ E. W. Ainley Walker and W. Murray, *Brit. Med. J.*, 1904, **2**, 16; R. Tunnicliff, *J. Inf. Dis.*, 1939, **64**, 59; A. D. Gardner, *Nature*, 1940, **146**, 837.

The conditions which favour the formation of these abnormally long cells are (*a*) the presence of certain drugs which inhibit division without inhibiting growth to the same extent—as when Ainley Walker and Murray obtained filaments by growing *Bact. typhosum* in presence of methyl violet or other dyes—and (*b*) the transfer of the cells to an unaccustomed medium to which the growth and division functions adapt themselves at different rates.

Under conditions which favour the long filaments, not only does the average cell length increase greatly, but the distribution of sizes becomes very much more scattered. It will be convenient now to consider in turn various detailed investigations which have been made on delayed division. For this purpose it is useful to introduce a quantitative measure of the size abnormality of any given culture. This has been done in the following way.† The sizes of cells are measured microscopically by comparison with a suitable ruled grid, the lengths being expressed as a number of arbitrary units. In a sample of a given population the number, ν_l, of cells having lengths in a unit range in the neighbourhood of l is then counted. The dependence of ν_l on l then gives the size distribution, the fact that the lengths are measured in terms of an arbitrary unit being immaterial. Since in a normal culture of the bacteria with which we shall be concerned, none of the cells exceed 2 units in length, we can measure abnormality by the function

$$\sigma = \sum_{3}^{\infty} \nu_l l.$$

For a normal population σ will be zero. With σ from 5 to 10 the size distribution is perceptibly abnormal under microscopic observation, and when σ is greater than 20 or 30 the field will present a rather remarkable appearance.

6. Filaments formed on transfer to new media

When a strain of *Bact. lactis aerogenes* which had been cultured for some time in bouillon was transferred to an artificial medium consisting of ammonium sulphate, glucose, magnesium sulphate, and a phosphate buffer the size distribution of the population was quite normal provided that the glucose concentration was high (38·5 g./l.). When, however, a small amount of the bouillon culture was transferred to a similar artificial medium containing glucose at one-

† C. N. Hinshelwood and R. M. Lodge, *Proc. Roy. Soc.*, B, 1944, **132**, 47.

twentieth the above concentration, cells of all sizes up to 20 or 30 times the normal length appeared.† σ (see last section) increased with n up to a maximum, after which it fell again, the culture sometimes, though not always, finishing up as a normal population. Some typical variations of σ with n are shown in Fig. 69.

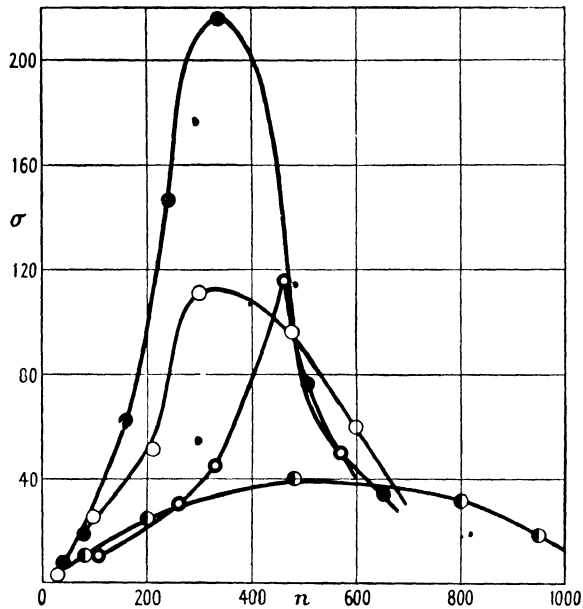


FIG. 69. Variation of size coefficient during growth cycle for various cultures giving filaments.

The production of the filaments is very clearly connected with the lack of adaptation to the new medium. When the cells are subcultured serially in the glucose-ammonium sulphate medium (whether the glucose is more or less concentrated) or even in a glucose-asparagine medium, the tendency to give long cells on transfer to the dilute glucose medium gradually disappears. The reduction of σ from over 100 (high degree of abnormality) to zero on serial subculture in the artificial medium is shown in Fig. 70.

In the artificial medium growth is slower than in bouillon. In accordance with general principles which have been discussed already,

† Hinshelwood and Lodge, loc. cit.

some degree of adaptation is necessary when transfer to this poorer medium occurs. If the division function is to some extent independent of the elongation function, the two may not adapt themselves at the same rate, with the result that a lack of balance leading to filament formation is created.

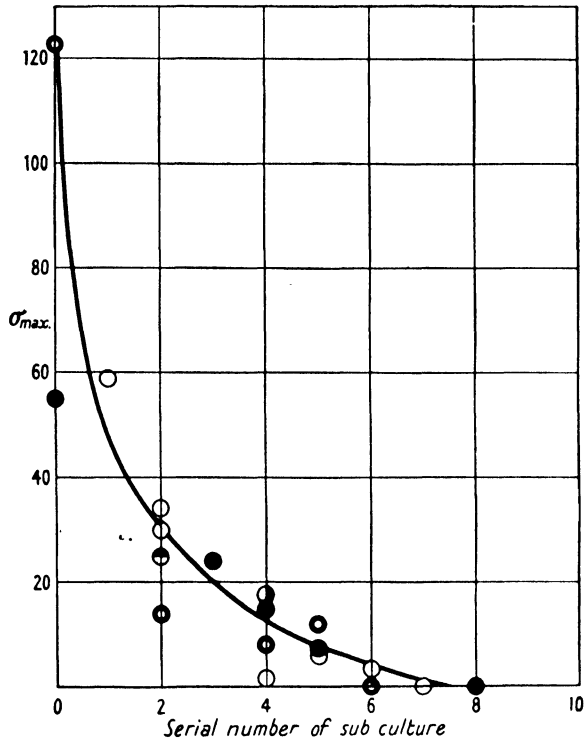


FIG. 70. Elimination of filament formation by adaptation to medium. The different kinds of point refer to various series of experiments.

It has been mentioned that filtrate from grown cultures of *Bact. lactis aerogenes* shortens the lag of young inocula, which means that it hastens the onset of growth. If division is not correspondingly hastened formation of filaments will be favoured, and it is in fact sometimes observed that the addition of the filtrate increases the tendency to give them. Various observations on the influence of inoculum age, inoculum size, and filtrate addition on σ can be correlated with the help of the hypothesis that two separate factors

favour elongation and division respectively, and that the former is diffusible into the medium. When the cells are transferred to a new medium, the rates of formation of the two factors, originally balanced, may become out of balance. Only when adaptation is complete is the balance restored.

The factors respectively favouring division and elongation may be referred to as *D* and *L*. They are subject to very specific influences. *D* appears to be formed much more readily in amino acid media than in ammonium sulphate media. In a glucose-asparagine medium filaments are never observed.

A certain rather atypical strain of *Bact. lactis aerogenes* (National Collection of Type Cultures 5268), characterized by very slow growth in the glucose-ammonium sulphate medium, showed a particularly marked tendency to give long filaments when grown for the first few times in it. If it was first grown in an asparagine-glucose medium, in which the *D* factor seems to be easily formed and consequently receives little stimulus from training, and then transferred to an ammonium sulphate medium, the lack of balance between the *D* and *L* factors became so marked that nothing but a tangled skein of thread of indefinite length appeared. Continued serial subculture eventually gave a normal population.†

Filament formation may also be observed when *Bact. lactis aerogenes* or *Bact. coli* are in the early stages of adaptation to other media, for example when *Bact. coli* is first transferred to an artificial medium in which ammonium sulphate is replaced by a nitrate as source of nitrogen. The tendency to give abnormally long cells is always lost as the adaptation proceeds. Filaments very similar to those formed by the coliform bacteria are also given under comparable circumstances by *Bacillus subtilis*. There seems to have been no special study of the way in which bacteria such as staphylococci, which are morphologically different from the coliforms or the bacilli, behave in these conditions, though analogous phenomena probably occur. An organism resembling *Staphylococcus aureus* was studied by Burke, Swartz, and Klise,‡ who observed on occasion coccus forms, rods, and filaments, which they regarded as parts of a definite 'life cycle'. They remarked that 'unfavourable environment interferes with the regularity of the life cycle, obscuring the stages and

† R. M. Lodge and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1943, **39**, 420.

‡ V. Burke, H. Swartz, and K. S. Klise, *J. Bact.*, 1943, **45**, 415.

resulting in a mixture of morphological forms'. Lack of balance between the functions of size increase and division would very probably explain these observations sufficiently well.

Though it is not referred to as producing specifically filamentous forms, *Corynebacterium diphtheriae* gives cells with abnormal morphology when it is transferred to new media.

A word should be said about the possibility that filaments are merely intruders of a foreign strain in an impure culture, the development of which is favoured by the new medium—a kind of suggestion which at one time was apt to be made in connexion with any manifestation of bacterial variability. Several answers may be made and seem conclusive. In the first place, filaments occur in cultures which have been carefully prepared from isolated single colonies. Secondly, the appearance with special prominence of an intruding strain would only occur in a new medium if this were such as to favour the growth of the filament-forming type at the expense of the normal one. Yet on serial subculture in the new medium this supposedly favoured strain disappears again. To explain this one would have to assume an advantage of the normal form, so that two contradictory assumptions have to be made to account for one set of facts. Thirdly, there is sometimes a definite mathematical relation between the sizes of all the cells in the population. This cannot be easily interpreted by the assumption of a foreign strain. Finally, in many circumstances the filaments may be observed to form and subsequently break up to yield a population of perfectly normal cells.

7. Size distribution in filament-forming cultures

As has been mentioned, in conditions which favour the formation of abnormally long cells the size distribution changes its character and widens very markedly. When *Bact. lactis aerogenes* is first transferred to the artificial medium containing dilute glucose, the following law is found to govern the distribution: if n is the total number of cells present, n_l the number with length greater than l, and $\bar{l}$ the average length, then

$$n_l = ne^{-l/\bar{l}}. \tag{1}$$

From equation (1),

$$\frac{d\ln(n_l/n)}{dl} = -\frac{1}{\bar{l}}. \tag{2}$$

† M. E. Maver, *J. Inf. Dis.*, 1931, **49**, 9.

Thus, if the law expressed by (1) is followed, $\log(n_l/n)$ plotted against l will give a straight line, the slope of which will be $1/2{\cdot}303\bar{l}$.

In Fig. 71 are plotted some typical results. Each line refers to a population sampled at a different stage of the growth cycle, as indicated by the values of n on the diagram (n is given in arbitrary but self-consistent units). Over a considerable range the exponential

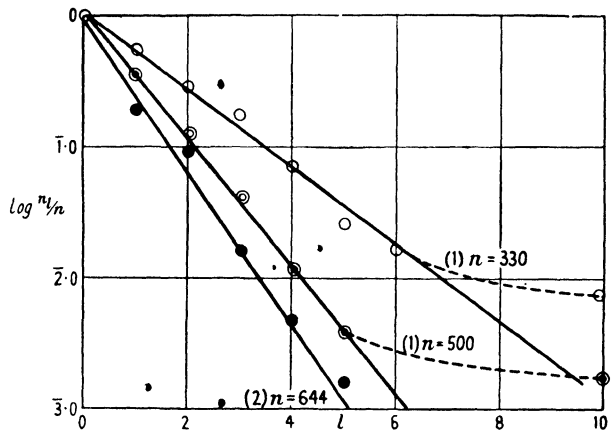


FIG. 71. Law of size distribution.

distribution law is quite well obeyed. There is, however, a quite definite departure from it in one respect, namely, that at certain stages of the growth cycle the number of exceptionally long cells is greater than would be expected, as is indicated by the course of the dotted lines in the figure. The number of cells of length up to about 5 times the average is rather closely given by the law, but the occurrence of those with 10 or even 20 times the average length is appreciably more probable than predicted by extrapolation. This appears to be not unrelated to another circumstance. Most of the long cells eventually split, and by the end of the growth cycle have become normal. In a population which has no member greater than about 10 times the average, the final distribution when growth is complete is indistinguishable from that of any ordinary culture. But if circumstances have allowed the appearance of cells of even greater length, then these often persist indefinitely and seem to be incapable of subsequent division. There may, therefore, be two kinds of influence at work, one which merely delays division and which determines the

number of moderately long cells, the other which comes into play when division has been too long delayed and which makes irreversible the initial disturbance of the normal processes. The influence of specific drugs on filament formation will be dealt with later, but in anticipation it may be mentioned that the deviation from the exponential law in populations formed under these conditions is very

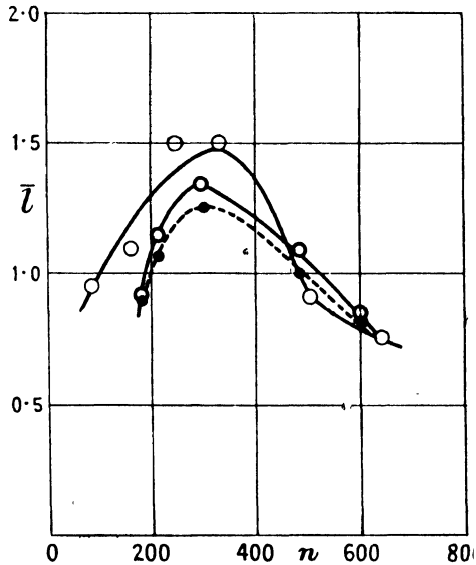


FIG. 72. Variation of mean size in culture cycle.

Open circles: inoculum 1, equation (2). Black-rimmed circles: inoculum 2, equation (2). Full circles: inoculum 2, equation (4).

much greater. With *Bact. lactis aerogenes* in the dilute glucose medium under the conditions described, the conformity with this law is, however, close enough to be regarded as of some significance.

The differing slopes of the lines in Fig. 71 indicate, in accordance with equation (2) that the average length $\bar{l}$ varies through the growth cycle. This variation may be examined in two ways, the comparison of which provides a means of subjecting the equation (1) to a further test. If we introduce the abnormal size coefficient defined in the last section

$$\sigma = \sum_{3}^{\infty} \nu_l l \qquad (3)$$

(ν_l being the number of cells in unit range in the neighbourhood of l)

ν_l can be related to n_l since the number of cells of length between l and $l+dl$ is $-dn_l$. Thus, in so far as sums over small finite elements may be replaced by integrals,

$$\sigma = -\int_3^\infty l\, dn_l,$$

and on substitution from (1) for dn_l we obtain

$$\sigma = n(3+\bar{l})e^{-3/\bar{l}},$$

whence

$$\bar{l} = \frac{3}{2{\cdot}303 \log\{(3+\bar{l})n/\sigma\}}. \tag{4}$$

Thus $\bar{l}$ can be found from σ, as well as from the slope of lines such as those in Fig. 71.

Fig. 72 shows that the two methods give concordant results, and also illustrates the variation of $\bar{l}$ during the growth cycle—which, in the examples taken, is complete at values of n in the neighbourhood of 1,000. $\bar{l}$ first rises and then falls again. The nature of the size distribution is such that relatively small changes in $\bar{l}$ are associated with very large changes in the frequency of the longer cells and in the abnormal appearance of the culture. Fig. 69 shows the actual variation during the growth cycle for some typical cultures of the quantity σ.

The exponential law which, in certain circumstances at least, governs the size distribution, is a common statistical form. A corresponding expression gives the number of molecules completing without collision a trajectory, in a gas, which is a particular multiple of the mean free path. The same form represents the probability that a railway which on the average has a disaster for every $\bar{l}$ miles of running shall achieve l miles without one. The basis of the law is that the events which terminate the undisturbed run, whether of cell elongation, molecular progress through the gas, or immunity of the railway from accident, shall be independent of the process which they interrupt. The chance of a lapse by a signalman (in so far as the law is to be legitimately applicable) is unaffected by the number of miles travelled since the last accident: the arrival of other molecules which interfere with the free flight of a given one is not conditioned by the length of path it has already travelled. If, therefore, the exponential law correctly describes the size distribution in the population of cells, it suggests that, under the circumstances prevailing, division is not determined by the size of the cell at the moment when

it occurs, but by the probability of certain independent events going on in the cell itself, or in its vicinity. This is not true in general (since, as we have seen, in normal circumstances the size distribution is narrow and not given by the exponential formula) but appears to apply when division is delayed on account of the imperfect adaptation of the cell to its environment. Normally, cell division attains a high degree of probability as soon as enough material has been synthesized: in special circumstances some conjunction of events independent of the actual length of the cell becomes the limiting factor.

If the probability of division becomes too small, the law of size distribution must change again (just as the distribution of free paths among molecules must change in a high vacuum), and when conditions are such that a large part of the bacterial mass appears as a tangle of threads, the limiting factor determining length must be something other than delayed division.

Another complicating factor is introduced by the possibility of the two kinds of delayed division which have been referred to in a previous section, namely, one where internal changes in the cell fail to occur and the other where these complete themselves duly but the cell nevertheless fails to split. The relation between the internal and the external changes is in need of further examination.

8. Inhibition of division by drugs

The observation by Ainley Walker and Murray† that *Bact. typhosum* forms filaments under the influence of dyestuffs has already been mentioned. The phenomenon occurs with other bacteria and with other inhibitors, and a number of scattered references to it occur in the literature. Most of the references in this section will be to work on a typical strain of *Bact. lactis aerogenes* about which more easily comparable observations are now available.

The action of the various growth-inhibiting agents is very specific. Proflavine gives fairly long filaments, but sulphanilamide, at least in a wide range of comparable circumstances, gives none.‡ *m*-cresol gives extremely marked long cell formation,§ but no trace of a corresponding effect can be induced by phenol. In a synthetic

† Loc. cit., p. 233.

‡ D. S. Davies, C. N. Hinshelwood, and J. M. G. Pryce, *Trans. Faraday Soc.*, 1944, **40**, 397.

§ G. H. Spray and R. M. Lodge, ibid., 1943, **39**, 424.

medium with dilute glucose ethyl alcohol gives normal forms, but tertiary butyl alcohol will produce very well-defined filaments.†

The substances which induce the abnormality are all powerful inhibitors of growth. Speaking once more of an elongation factor, L, and a division factor, D, it is a question with a given drug whether L or D is the more adversely affected. If L suffers more serious reduction than D, then there will be no filaments. Since we have to deal with a balance between two influences, it is not surprising that the occurrence of the filaments, even with a particular inhibitor, may depend rather specifically upon the conditions of working. Spray and Lodge found that to obtain the most striking results with *m*-cresol it was necessary to use concentrations approaching those which would inhibit growth completely, and also to transfer the inoculum from a parent culture of such an age that the lag in the new medium would have been almost zero in the absence of the drug.

It is of interest in connexion with ideas on the influence of surface tension on cell division to note that surface active agents such as cetyl trimethylammonium bromide and sodium dodecyl sulphate seem to have little effect in stimulating any abnormal size distribution.‡

When the cells adapt themselves to resist the action of the drug, the power of the latter to induce filament formation vanishes. If, in untrained cells, a concentration m_1 of proflavine induces it, then after training to $\bar{m}$, there will be none, even under the influence of concentrations much greater than $\bar{m}+m_1$.§ The mechanism of division is here, apparently, even more easily adapted than that of growth.

Bact. lactis aerogenes does not adapt itself to resist phenols, and, accordingly, does not lose its susceptibility to the morphological disturbances caused by *m*-cresol. Indeed, so far from this, Spray and Lodge found that after filaments had been produced under the influence of the cresol, these continued to form, even on repeated subculture in a medium free from inhibitor, the power of division having suffered an impairment from which recovery proved to be extremely slow.

In the later stages of growth the filaments formed under the

† C. N. Hinshelwood and R. M. Lodge, loc. cit., p. 234.
‡ A. R. Peacocke, unpublished observations.
§ A. M. James, in the press.

influence of drugs tend to give place to normal forms, as occurs in the absence of drugs but, especially when the distribution has been very abnormal, many of the longest cells may persist. A culture grown under the influence of proflavine, in conditions such that few cells in excess of 10 times the normal length appear at any stage, is often normal by the end of the growth cycle, but with one grown under the influence of cresol and showing little but cells 20 to 50 times the usual length, the abnormal size distribution is generally persistent.

When a long cell breaks up it seems ω do so not by shedding segments one by one from its end, but by a more or less simultaneous series of divisions along its whole length. This matter is, however, in need of closer study.

9. Influence of osmotic pressure

It has been mentioned that *Bact. lactis aerogenes* shows delayed division when first transferred to an artificial medium containing dilute glucose, but that if the glucose concentration is high, the size distribution is normal. The highest value, σ_{max}, to which the size coefficient attains during the growth cycle proves to be a function of the osmotic pressure of the medium. This can be shown by the addition to the dilute glucose medium of various other substances such as sodium chloride, ammonium sulphate, or erythritol (representing a substance chemically similar to glucose but not utilized by the bacteria). These all cause a reduction in σ, and, what amounts to the same thing, of $\bar{l}$. The reduction is approximately proportional to the increase in osmotic pressure as shown in Fig. 73. These results suggest that the influence of the glucose concentration itself is primarily an osmotic one. The curves in Fig. 74 show the variation in σ during the growth cycle for various initial concentrations of glucose (expressed as fractions of the highest, 38·5 g./l.). For the concentration 1 (the highest), σ is zero throughout. The marked rise as the glucose concentration drops to $\frac{1}{10}$ of this is very evident. At lower concentrations still, σ_{max} seems to fall again, but this effect is probably illusory. Below 0·1 the total population which the medium will support decreases rapidly. At 0·025, for example, n_{max} is 209 and reference to the figure shows that σ_{max} is only reached at this point. If growth had been able to go on, σ would probably have risen still higher. Indeed, in the figure the maxima from 0·01 to 0·1

appear to have a common envelope, which suggests that if n could increase further σ would do so also.

When the formation of filaments is caused by proflavine or by m-cresol, addition of enough sodium chloride to the medium completely suppresses them, and the maximum value of σ observed at any stage of the growth cycle is zero. Small additions of sodium

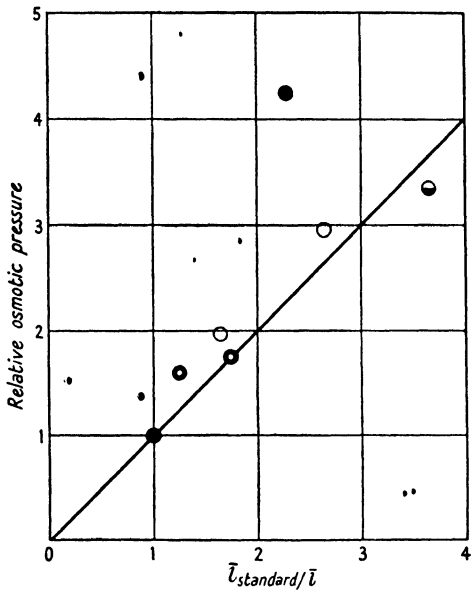


FIG. 73. Influence of osmotic pressure.

chloride, however, actually enhance the filament formation.† These effects are shown in Fig. 75, where the abscissae represent the weights of sodium chloride added to each culture tube containing 26 c.c. of the synthetic medium.

10. Influence of temperature on delayed division

Increase of temperature has a pronounced effect in favouring the formation of the filamentous cells. All the results which have been described in the foregoing sections were obtained in experiments at 40°, at which temperature the growth rate is at its maximum value. At 30° the size distribution becomes much more nearly normal, even

† A. M. James, in the press.

when growth occurs in presence of high concentrations of cresol or proflavine. The change in the value of σ with the temperature of growth is shown in Figs. 76 and 77 in which some results found by A. M. James are plotted.

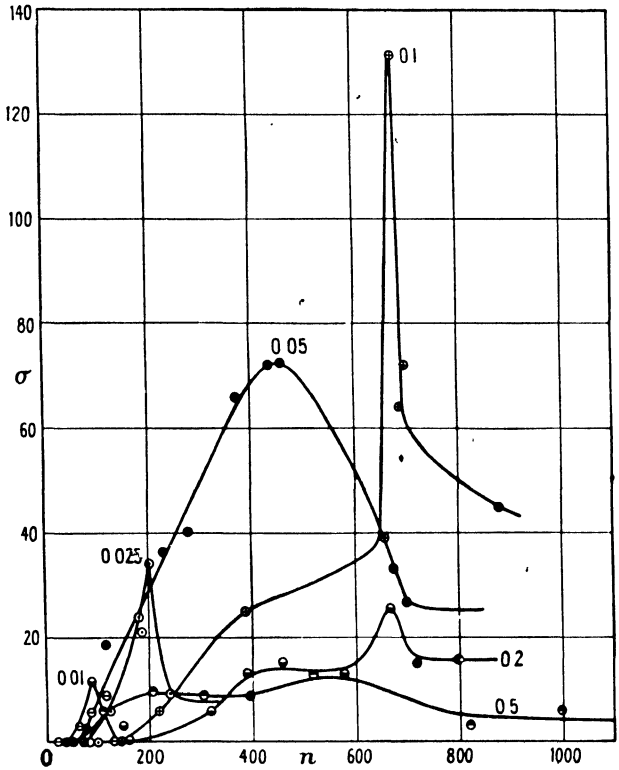


FIG. 74. Variation of σ with initial glucose concentration.

In this connexion certain adaptive effects become apparent. Cells which have been subcultured for some time at 20° show much more susceptibility to filament formation when grown in presence of drugs than those which have been acclimatized by serial subculture at 40° before exposure. This is illustrated by the curves in Fig. 77. No appreciable adaptive changes in overall growth rate are detectable with these bacteria in experiments where they are trained by serial subculture at one temperature and then tested in successive growth

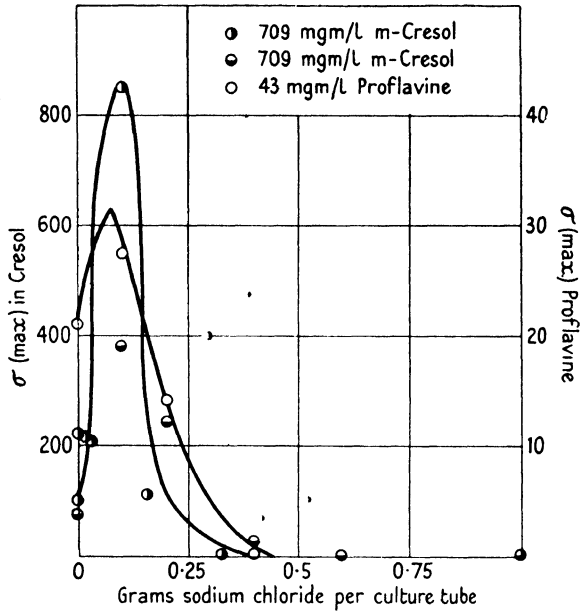


FIG. 75. Influence of salt additions on production of filaments by cresol or proflavine.

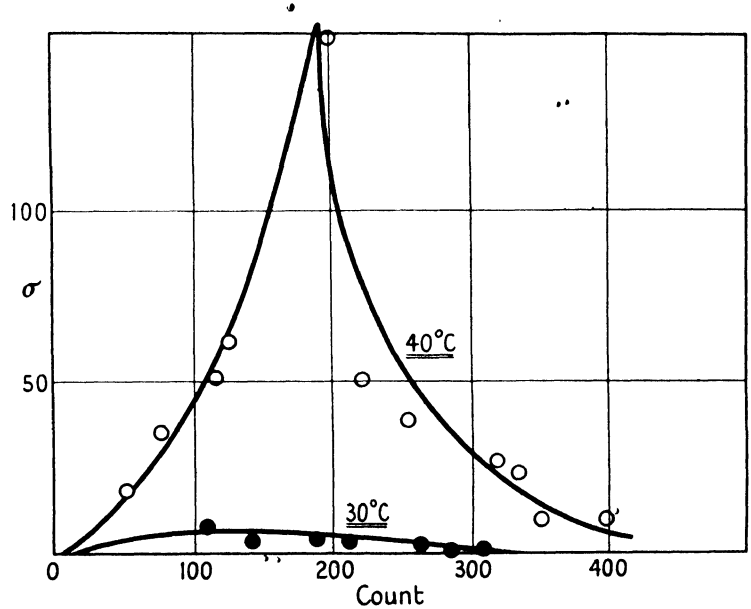


FIG. 76. Influence of temperature on filament formation.

cycles at other temperatures, though such changes must, in principle, occur. But the more delicate balance of what we have called the *D* and *L* factors seems to provide a subtler means of revealing such adaptive processes.

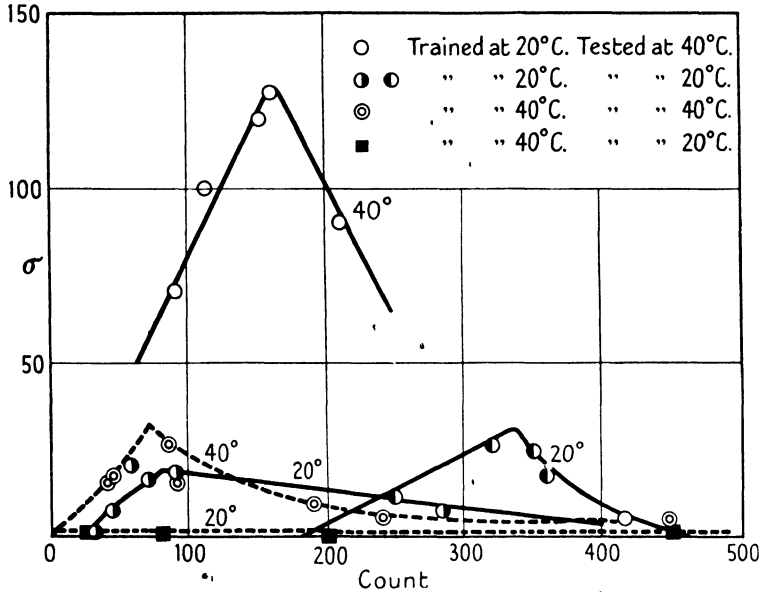


FIG. 77. Influence of temperature and of temperature-adaptation on filament formation.

11. Size distribution in cultures grown under the influence of inhibitors

With *Bact. lactis aerogenes* and proflavine the sort of distribution found is illustrated in Fig. 78: there is some tendency to conform to the logarithmic law, but the proportion of longer cells is rather greater than this law would predict, as is evident from the fairly marked curvature of the lines in the diagram.

With *m*-cresol the distribution is quite different, and there is something more like a tendency for the bacterial mass to be equally distributed in all the size ranges, as though very long threads had been snapped at random by mechanical action.

12. Initiation of the division process

In the course of the foregoing various references have been made, in a general and unfortunately not very precise way, to the operation

of the scale effect as the key to the division process. According to this a constant relation between the various chemical and physical factors cannot be maintained as the ratio of the area to the volume changes. The work of Robinow and the analogy of cells showing

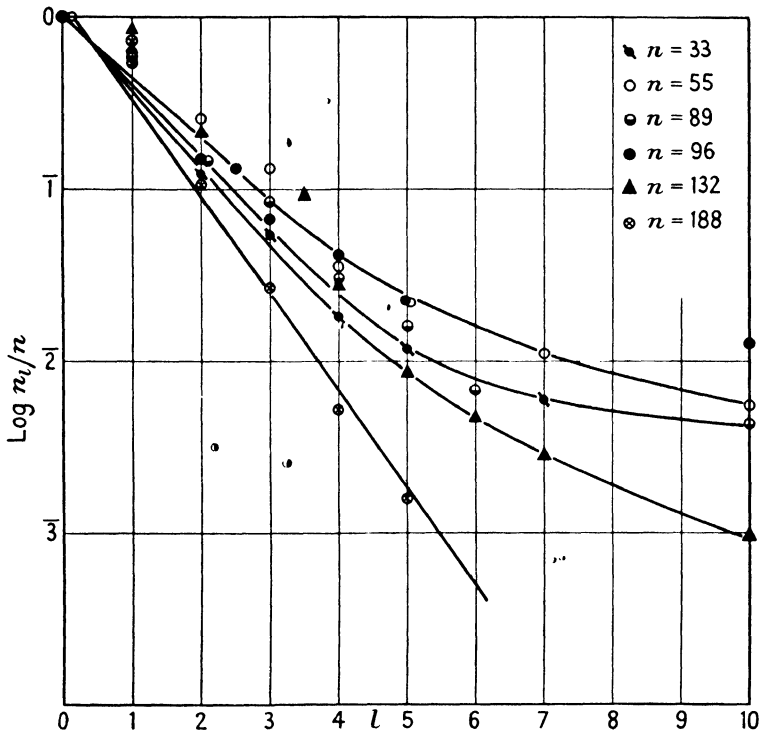
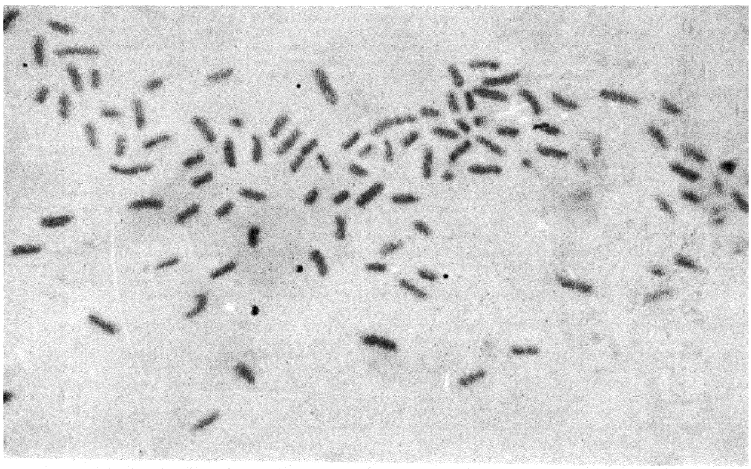


FIG. 78. Size distribution of filaments formed by proflavine.

definite mitotic phenomena suggest that the initiation of division occurs in some specialized structures in the interior. If this is so there are still two possibilities: the splitting of these structures themselves may be determined by their own geometry and energy relations or by those of the whole cell. Their own size governs physical factors such as mechanical strength, while the surface-volume ratio of the cell as a whole regulates chemical factors such as the concentration of substrates arriving from outside or of metabolites diffusing away from inside. These chemical factors in their turn influence the

surface energy and indeed the actual material composition of each special part of the cell.

The observations on abnormal division which have been discussed in the last few pages suggest that the chemical factors are of primary importance. It seems that separate elongation and division factors can be defined. It also seems that the latter are modifiable by drug action, and by the change in enzyme balance which accompanies adaptation. The working hypothesis that critical concentrations of certain chemical substances have to be built up before the cell divides would account for some of the facts—although, to be sure, it would leave plenty untouched. The attainment of the threshold concentration could obviously be impeded by specific drug actions: it would certainly depend upon the proper adaptation of the manufacturing enzymes. And it would probably be hastened by an increased osmotic pressure in the medium, which would cause withdrawal of water from the cell and so increase the internal concentrations of metabolites. Furthermore, if precipitation of the dissolved material had to occur, there would be variable and irregular delays of the kind observed to attend division. The delays in question would only have a serious effect on the cell morphology when the *D* factor was kept in short supply. Normally the supersaturation would rise so quickly that elongation would appear to be the determining factor. Thus there would be a narrow size distribution when division was favoured and a broad one when it was impeded. This also would correspond to the experimental findings.



1

2

BACT. LACTIS AEROGENES

1. Normal cells; 2. Filaments formed by inhibition of division

(The magnification in (1) and (2) is the same)

XI

OTHER EVIDENCE RELATING TO THE NATURE OF CELL ORGANIZATION

1. Introduction

In this chapter several matters will be briefly discussed which raise questions of considerable physico-chemical interest, without, however, providing complete or definite answers to them. The problems are rather diverse ones, though they have in common that in all of them bacteria manifest themselves as labile colloidal systems. Each topic might well form the subject of a complete chapter, but here we shall only deal with certain specific aspects which are germane to our general theme.

2. The death-rate of bacteria

Unless they are rejuvenated by division, bacterial cells eventually die, the death-rate being increased by high temperatures, antiseptic substances in the medium, and exposure to radiations such as X-rays or ultra-violet light.

Great interest has been aroused by the law according to which the number of survivors decreases with time in a declining population. Many observers have expressed their results by the simple exponential formula,

$$N = N_0 e^{-lt},$$

where N is the number of survivors at time t out of an initial N_0, and l is a constant. This equation gives the familiar die-away curve shown by the line I in Fig. 79. If the results are indeed correctly representable by this curve, then conclusions of considerable interest follow, but it seems to be by no means generally agreed that they are.

The most convenient order of discussion will be first to consider the implications of the exponential law, assuming for the moment that it does do justice to the facts. The form is of course similar to that giving the amount of unchanged substance in a unimolecular reaction, and this similarity has unfortunately led to misunderstandings on the part of some upholders of the law (and even worse ones on the part of their critics, when the latter have said that since the death of bacteria cannot be a unimolecular reaction, the exponential law must be meaningless). The meaning, if the law applies, is in fact

quite definite, and has nothing to do with unimolecular reactions. The conditions that it should apply are, first, that the death of a given cell should not be influenced by the number of other survivors (as it would be, for example, if there were a competition for residual food material), and, secondly, that the chance of death in a short interval of time, *dt*, is independent of the previous history of the cell. This is where the interesting implications come in. Suppose that

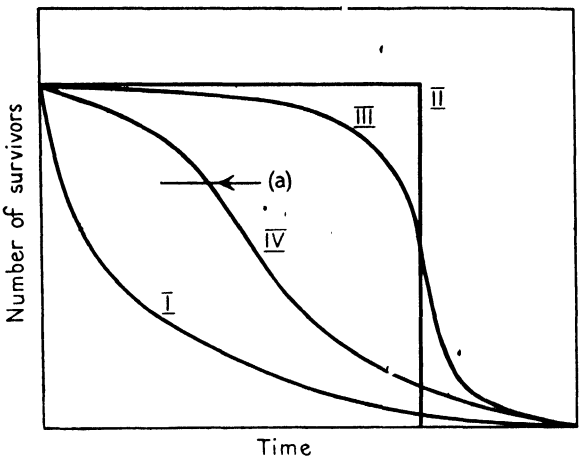


FIG. 79. Death-rate of bacteria according to various hypotheses.

death is caused by the presence of an antiseptic (about which there would be no doubt since the cells would not die in its absence), and yet is independent of the time of exposure. This would mean that at a given moment there occurs some chance conjunction of events suddenly exposing the cell to a lethal action which has not so far affected it.

Two kinds of event are conceivable. One is a chance encounter between the lethal agent and some sensitive part of the cell which is too highly localized to be found except on rare occasions. The other is some spontaneous internal change in the cell organization, which occurs without reference to the presence of the lethal agent, but which, when it once happens, exposes the cell to the action of that agent. The former kind of happening has been suggested in explanation of the action of X-rays on the cells of *Bact. coli*, which are assumed to be killed when a quantum of the radiation collides

with some localized sensitive site in the bacterium.† The conception of a quantum of radiation as a physical missile is quite an acceptable one from the theoretical point of view, but the application of the corresponding interpretation to drug action is less satisfactory. The alternative idea suggests interesting possibilities, namely, that there are certain moments at which cells are specially sensitive to the action of drugs or of radiation. This would mean that the internal condition was far from static, even in a cell not undergoing active division. In this connexion it is perhaps relevant to mention that young cells are stated to be considerably more sensitive than old ones.‡

The whole idea upon which the foregoing discussion is based is challenged by some writers, who maintain that the action of the drug (or by implication, even if not explicitly stated, of the radiation) is not independent of the time of exposure.§ One might certainly have expected the chances of death to increase with the time of exposure to the adverse environment. If the death of the cell occurred after exposure for a perfectly definite time, the number of survivors would vary with time as in curve II in Fig. 79. But inequalities among members of the population are inevitable: some will survive for a longer and some for a shorter time than the average, so that the curve II will be rounded off to give curve III, or even more to give curve IV. By assuming a very special distribution of natural resistances, one could even account for a curve of strictly exponential form, that is, curve I. This, however, would mean a highly improbable initial distribution. Instead of a maximum proportion of cells with a survival time somewhere near the average, one would have to assume that the most frequent survival time was nearly zero, and that the frequencies of the others fell off in just the way required to give curve I.

At this stage we must return to the question which was deferred at the beginning of the section, namely, whether the experimental curves are in fact of strictly exponential form. In some cases it seems clear that they are not.‖ Moreover, it is pointed out that under the

† D. E. Lea, R. B. Haines, and C. A. Coulson, *Proc. Roy. Soc.*, 1936, B, **120**, 47; 1937, **123**, 1; J. A. Crowther, ibid., 1926, B, **100**, 390; D. E. Lea, *Actions of Radiation on Living Tissues*, Cambridge, 1946.

‡ This, however, includes sensitiveness to heat: C. N. Stark and P. Stark, *J. Bact.* 1929, **18**, 333.

§ Cf. A. J. Clark, *General Pharmacology.*

‖ See Clark, loc. cit.; E. R. Withell, *Quart. J. Pharmacy and Pharmacology*, 1938, **11**, 736.

conditions of many experiments a considerable proportion of the organisms are already dead by the time the first measurements are made. This means that the observation of a curve such as IV in the figure would begin, not at the origin, but at a point like (*a*), and the result of this would be to make a sigmoid curve more easily mistakable for an exponential curve. There is certainly a good deal of weight in these contentions, but one has, nevertheless, the impression that in certain examples, especially those where the action of radiation is involved, the exponential law is too good an approximation to be explained away simply by a special kind of distribution of sensitivities.† There is probably room for more experimental work on this matter, the theoretical issues which it raises being of the first importance.‡

3. Influence of temperature on the growth of bacteria

In lower temperature ranges the rate of growth of bacteria increases rapidly with increase in temperature, the change being about two- to threefold for 10°. A curve of mean generation time as a function of temperature for *Bact. coli* was determined by Barber,§ and shows the following characteristics. Up to 35° the generation time drops, between 35° and 45° it remains almost constant, and between 45° and 50° it rises very steeply. At higher temperatures growth fails entirely, and the cells are killed. Later experiments made with *Bact. lactis aerogenes*, which shows quite similar behaviour, added the observation that from 25° upwards the total population which the medium supports decreases with accelerating rapidity and becomes zero at about 46°.‖

Since lag is a function of the age of the cells, and at an appropriate value of the latter passes through zero, its variation with temperature cannot be simply expressed. *Bact. lactis aerogenes* which shows no lag at 20° also shows none at 40°. Older cultures which have a long lag at 20° have a shorter lag at 40°, but the diminution with temperature of the lag is *less* than that of the mean generation time over the same range (A. M. James).

Since growth depends upon a sequence of reactions, one must

† Moreover, as Lea points out (op. cit.), various technical errors can distort a curve which should be exponential into a non-exponential form.

‡ Cf. R. C. Jordan and S. E. Jacobs, *J. Hyg.*, 1944, **43**, 275.

§ M. Barber, *J. Inf. Dis.*, 1908, **5**, 379.

‖ R. M. Lodge and C. N. Hinshelwood, *J. Chem. Soc.*, 1939, 1683.

expect a certain complexity in the law of temperature variation. Nevertheless, the analogy of unorganized systems leads to the expectation that there should be an approximate accordance with the Arrhenius law, and this is in fact in evidence up to about 25°. The subsequent passage of the growth rate through a shallow maximum and the final catastrophic decline to zero must depend upon the intrusion of a quite foreign factor. This factor is clearly the degeneration of the cell organization, which is closely connected with the denaturation and coagulation of the proteins. The latter process is well known from independent experiments to have a very high temperature coefficient, and in consequence inactivation of enzymes and the thermal degeneration of cells set in and become complete over a comparatively narrow range of temperature.

If we write:

effective rate of growth = rate of synthesis—rate of degeneration,

then the first term on the right-hand side will be expressible at least approximately as $A_1 e^{-E_1/RT}$ and the second as $A_2 e^{-E_2/RT}$, where E_2 will be very considerably greater than E_1. The effective growth rate is then approximately of the form:

$$A_1 e^{-E_1/RT} - A_2 e^{-E_2/RT}.$$

At low temperatures the second term is negligible and the rate increases in approximate accordance with an Arrhenius equation, with an activation energy E_1. Over a certain range of temperature the two terms will be of the same order of magnitude, and in a quite narrow region will nearly cancel one another. After that the negative second term far outweighs the first, and the rate falls very rapidly to zero.

One rather interesting inference may be drawn here. The growth rate of *Bact. coli* changes very little in the range 35° to 40°. Now the actual rate of synthesis is quite high at 35° and the temperature coefficient at lower temperatures shows that E_1 is fairly high. At 40°, therefore, one concludes that the degenerative processes must be quite active, being able to neutralize the effect of what would have been quite vigorous synthesis. Yet, at this temperature the loss of activity of enzymes in non-growing cells, although quite measurably rapid, is slow compared with the growth rate. Thus the degenerative processes seem to act upon cells in which the manufacture of substance is occurring more effectively than they do on

resting cells. This is in a general way quite understandable, the labile intermediate configurations which must appear during growth being more susceptible to denaturing influences.

The degenerative changes which occur under the influence of increased temperature are themselves of some interest from the point of view of chemical kinetics.† Denaturation of proteins and inactivation of enzymes show exceptionally high activation energies, up to 100,000 calories or even more. These correspond to the extremely high temperature coefficients which determine the apparent suddenness with which the processes supervene when the temperature is raised. On the other hand, they do not prevent the processes from attaining a measurable velocity at quite low temperatures, such as 45–60°. A chemical reaction of simple molecules with an activation energy of 100,000 calories would not normally be observable at temperatures lower than many hundreds of degrees.‡ The non-exponential factor of the Arrhenius equation must therefore be exceptionally high for the processes now in question. This factor corresponds to the entropy of activation. When it is large, it means that the activated state of the molecule has a very high degree of probability relative to the initial state. Thus, we infer that the activated state must be highly disordered compared with the initial state. This results in an easy transition to the activated state in spite of the large amount of energy which has to be taken up to reach it. It is precisely in such highly ordered systems as undenatured proteins, or the other structures of living cells, that there is room for very large entropy increases when the configuration is destroyed.

We thus remain with the following picture of the temperature relations of the living cell. At low temperatures there is a very high degree of extremely specialized order (which preserves from destruction all kinds of active catalytic centres, possibly structures with free radical ends, and so on). Synthetic chemical reactions leading to growth occur with moderate activation energies. The ordered configuration is stabilized to some extent by the energy relationships, which are such that considerable work has to be done to disturb the well-organized pattern. This means a high activation energy for the processes of destruction, which at lower temperatures are therefore

† S. Glasstone, K. J. Laidler, and H. Eyring, *The Theory of Rate Processes*, 1941, p. 442; H. Eyring and A. E. Stearn, *Chem. Rev.*, 1939, **24**, 253.

‡ C. N. Hinshelwood, *Kinetics of Chemical Change*, 1945.

quite insignificant, and would remain so up to much higher ones, were it not for the high entropy factor, expressing the great tendency of nature to proceed to a state of disorder. In a certain narrow range of temperature the destructive processes catch up with and overtake the constructive, after which, in virtue of the high activation energy of the former, they rapidly prevail.

In the denaturation of various proteins and the inactivation of certain enzymes there is a compensation between the energy and entropy factors which leaves the respective rates of reaction rather close to one another.† For example, for egg albumen, haemoglobin, pepsin, rennin, and trypsin the activation energies vary from 40,000 to 132,000 calories, in spite of which the free energies of activation (that is quantities derived from the logarithm of the reaction rate) only vary from 21,900 to 25,700. The data, however, have been selected by nature, in that all the substances involved are associated with warm-blooded animals. With a wider ranging selection, more diverse behaviour might be expected. One can well conceive structures, in which the energy-entropy relationships are such that degenerative processes do not set in before quite considerably higher temperatures are reached. There is no need to suppose structures of which the chemistry is fundamentally different. With energies ranging as for those just mentioned, and entropies in the same range; but with different combinations of the individual values, quite diverse free energies and reaction rates could be arrived at. This being so, it is perhaps not surprising that various kinds of cell do in fact exist, capable of living at considerably higher temperatures than those which bring about the death of the more familiar bacteria. These are, on the one hand, the so-called thermophilic bacteria, and, on the other hand, bacterial spores.

4. Thermophilic bacteria‡

Bacteria such as the coliform group, staphylococci, streptococci, and so on, are killed by exposure to temperatures of 50° to 60° C.

The thermophilic group grow readily and perform their biochemical functions at 70° C. They are found in nature in hot springs, sewage,

† Glasstone, Laidler, and Eyring, loc. cit.

‡ See A. A. Imshenetzky, *Microbiological Processes at High Temperatures* (Микробиологические Процессы при Высоких Температурах), Academy of Sciences, U.S.S.R., 1944.

the intestinal contents of various animals (from which they find their way into manure), in the air, in milk, and in the soil.

On the whole, their biochemical activity resembles in general character that of bacteria with lower temperature optima. But in virtue of the fact that they can function at considerably higher temperatures, the maximum rate at which they can perform such operations as proteolysis, denitrification, hydrolysis of starch, and fermentation of cellulose is very substantially greater than the corresponding maximum for the non-thermophilic groups. This is obviously a matter which can assume practical importance. The high degree of chemical activity is closely related to a very rapid rate of multiplication, also a simple consequence of the higher temperature range.†

5. Bacterial spores

Thermophilic bacteria resist heat and are characterized by chemical activity corresponding to the higher temperatures at which they flourish. The cells of certain genera of non-thermophilic bacteria (*Bacillus* and *Clostridium*) have the property of changing into a modified morphological state in which they are known as spores. The spore is very much more resistant to heat than the bacterium, and will stand temperatures over 100° C., but it possesses little chemical activity, and is usually referred to as a resting form of the organism. It is not only resistant to heat but to desiccation and the action of antiseptics, and thus possesses great power of survival in adverse circumstances. It is sometimes stated that spores are formed in response to unfavourable environment, but this is not correct, and, indeed, with some species spore formation requires rather nicely balanced conditions. One of these is that the food supply must be running out, not because this constitutes an adverse circumstance against which the organism protects itself, but because otherwise any spores formed would at once germinate again to yield normal cells.

When a spore is produced there first appears inside the cell some granular matter which possesses modified staining properties. This matter grows into a spheroidal mass surrounded by a sort of halo

† A simple physical calculation soon disposes of the suggestion that thermophilic bacteria are protected against the effects of high temperatures by a non-conducting sheath. The thermal conductivity of the latter would have to be inconceivably smaller than anything known.

which presently becomes the tough spore membrane. The whole spore may be near the end or near the middle of the original cell. The spores of a given species seem to possess rather different degrees of heat resistance, different staining and refractile properties, and different sensitivities to antiseptics according to the completeness and perfection with which the transformation has completed itself.

The whole question of spore formation is of the greatest interest in that it shows the normal chemical constituents of cells to be capable of regrouping in such a way as to give configurations which do not suffer denaturation at temperatures where this normally occurs. The regrouping apparently spreads from certain nuclei in the cell, and the new kind of organization caused by this elaborate polymorphic change seems to induce the formation of a new kind of cell wall as soon as a large enough material mass has been involved in the transformation. In these respects the process of sporulation depends upon the same kind of material mobility of the internal structural elements of the cell as is involved in actual division. The spore might be regarded as an alternative pattern which the essential macromolecular components of the cell can form while still in an active enough state to be capable of rearrangement. The alternative arrangement possesses greater stability and correspondingly lower activity. The regrouping may well be found to be catalysed or inhibited by specific agents just as division appears to be. Prazmowski observed with *Clostridium butyricum* that some cells might be multiplying while others were forming spores, and Stephenson and Cook made similar observations with *Bacillus subtilis* and with *Clostridium sporogenes*.† R. M. Lodge and the writer with a strain closely related to *Bacillus subtilis* observed that forms having at least some of the properties of spores seemed to be thrown off in almost constant proportion during most of the logarithmic growth phase, and that only in a quite narrow region of the growth cycle as the lag merged into the logarithmic phase was the culture susceptible to complete sterilization by heating to 90° C.

If one assumes that spore formation requires a good deal of internal activity in the cell and is likely to occur in much the same circumstances as division, it is clear none the less that the conditions for active growth with synthesis of new substance will keep on regenerating the normal bacterial structure. Germination of spores

† See R. P. Cook, *Biol. Rev.*, 1932, **7**, 1.

to give ordinary cells does in fact occur as soon as conditions favour the synthetic reactions. This may well be the reason why the degree of spore formation is so erratic. Many observations exist on the percentage of spores in the total population when all activity in the culture has died down, and many attempts have been made to define the influence of various factors on this proportion, but the results seem to be rather confusing. If we need a high degree of metabolic activity combined with the cessation of further germination, it is evident that whether or not a given cell is finally left in the spore form will be a very delicately balanced matter.

There are doubtless other unknown factors, the significance of which we are not in a position to assess, and the subject will not be discussed further here.† It should be remarked, however, that the whole question is one which ought ultimately to throw much light on bacterial organization.

6. Adaptation of bacteria to changed conditions of temperature

The rapid and easy adaptation of bacteria to resist many drugs, or to utilize new sources of material, raises the question of the extent to which they can be acclimatized to new conditions of temperature. This form of adaptive process proves, however, to be extremely slow and difficult, if not often impossible.

The earlier literature contains several accounts of researches on the artificial cultivation of heat resistance. Dallinger (1887) exposed certain protozoa to gradually increasing temperatures during seven years and trained them to tolerate 70° C. after they had initially been unable to stand more than 23°.‡ Dieudonné§ by gradual transfers to higher temperatures raised the maximum which could be withstood by 4° to 5° for various strains of bacteria, but nothing comparable to the transformation of a non-thermophilic bacterium into a thermophilic one has ever been achieved.

Casman and Rettger‖ subcultured several kinds of bacteria daily for about a year at their respective temperatures of maximum growth, and from time to time attempted to raise the temperature by 0·25°

† Reference should, however, be made to the discussion of I. Kaplan and J. W. Williams, *J. Bact.*, 1941, **42**, 265, where some of the views expressed may seem to differ rather widely from those indicated in this section—though possibly more in form than substance.

‡ W. Dallinger, *J. R. Micros. Soc.*, 1887, 185.

§ A. Dieudonné, *Centralbl. f. Bakt.*, 1894, **16**, 965.

‖ E. P. Casman and L. F. Rettger, *J. Bact.*, 1933, **26**, 77.

to 0·5°. During this treatment the cultures frequently died, and at the end of the time there had been only a negligible degree of acclimatization with *B. subtilis*, *B. mesentericus*, *B. megatherium*, *B. cereus*, and other strains. The following are a few typical results:

	Maximum growth temperature	
	Trained	*Control*
Bacillus subtilis	59–60	59–60
Bacillus cereus	49	47–9
Bacillus megatherium	47	43–4

Casman and Rettger remark that the relatively short period of acclimatization does not allow a definite conclusion that there is no adaptation, but they point out that the process is very much more difficult than they had been led to expect from the reports of other investigators. This raises the question whether some of the earlier more positive results were perhaps due in part at least to a concomitant adaptation of the cells to the growth medium itself.

Rettger and others have also correlated the maximum growth temperature of various kinds of bacteria with the temperature of destruction of different enzyme systems contained in the cells, and conclude that the limitation of growth depends primarily upon the inactivation of the enzymes responsible for cellular respiration.†

Imshenetzky,‡ after experiments lasting for about 18 months, came to the same conclusion as Rettger and Casman. With acetic acid bacteria he obtained negative results, degeneration of the cell substance setting in when the temperature was raised: and the findings with *Bacillus felsineus* were somewhat similar. With *Bacillus mesentericus* he succeeded in raising the maximum growth temperature from 53° to 55°, and also showed that the proteolytic activity of the trained strain at 55° was several times as great as that of the original strain at this same temperature. With yeasts he also obtained certain positive results. On the whole, however, he concludes that the process of adaptation to higher temperatures is quite unlike that of adaptation to drugs: it is very much slower and much more restricted in scope. Nothing analogous to the throwing off of thermophilic variants from normal cells is ever observed, and if the kind of bacteria which resist 70° C. are to be formed from those which are killed by 50°, then this must involve a process of a higher order

† O. F. Edwards and L. F. Rettger, *J. Bact.*, 1937, **34**, 489. ‡ Op. cit.

of improbability than those normally observable in laboratory experiments.

From the theoretical point of view one can see in a general way that adaptation to temperature must be a process involving much more profound changes in the cell material than those which accompany training to drugs or to new substrates. The latter seem to depend upon a simple change in the quantitative balance of the enzymes, or, possibly, rather subtle modifications in the actual texture of the enzyme substance. The development of greater temperature resistance, on the other hand, involves a complete reorganization of the cell structure so that the energy–entropy relationships of the proteins—and indeed the whole material pattern—become quite different (see discussion of maximum growth temperature in § 3). Thus adaptation to drugs or substrates can occur by a gradual modification which accompanies growth, while, it would seem, adaptation to high temperature would require the imposition of a new kind of organization. This, of course, is precisely what happens in the process of sporulation, except that spore formation is accompanied by loss of chemical activity, and, moreover, occurs spontaneously rather than in response to an increased temperature of the environment. For the various cell configurations to acquire the appropriate stability without the corresponding loss of activity, thus giving a thermophilic variant, the modification required seems to be nearly as profound as that which would be demanded for a complete change of species. Bacterial variation has never reliably been observed to result in transformations as deep-seated as this.

While the acquisition of substantially increased heat resistance would involve very radical changes in the cell, there is another form of adaptation to temperature which should, in principle, be observable. Since the rate constants of the various synthetic processes in the cell presumably have somewhat different temperature coefficients, one would expect the exact proportions of the various enzymes to depend upon the temperature at which the cells are grown. Further, since a new enzyme balance can only supersede an existing one after a considerable renewal of cell substance, one might expect cells grown at one temperature and then subcultured at another, only gradually to show the true equilibrium growth rate characteristic of the latter. Actually, the effects to be expected depend upon the *differences* in the temperature coefficients of various processes and

not on these coefficients themselves, and the results seem to be difficult to detect. *Bact. lactis aerogenes* has been stabilized at various temperatures in serial subculture, and then tested at others. The growth rate (and the lag) were in general found to be determined solely by the actual temperature of the test, and to show little, if any, detectable influence of the adaptive history.† An exception to this has already been mentioned (p. 246): the delicate balance of the factors determining cell elongation and cell division is observably influenced by the thermal history, cells which have been trained at a low temperature giving filaments much more readily on transfer to a higher one, than cells which are already adapted to the latter.

7. Lysis of bacterial cells

A fully-grown culture of *Bacillus subtilis* may suffer almost complete autolysis after a few days, with destruction of the cells and the appearance in the medium of breakdown products of proteins. Similar phenomena occur with other bacteria, but to very varying extents, none, for example, being observable with *Bact. coli* after many days. The ease of the lysis is a function of the original conditions of growth.

The destruction of the bacterial substance is brought about by the action of special autolytic enzymes, which become free to operate upon the cell substance in general as soon as the organization of the system begins to break down, and is no longer able to hold them spatially separated from material which they can decompose.

Bacteria may also suffer lysis under the attack of agents known as phages. These are related to viruses, and can be obtained from sources such as sewage. They exert a lytic action which is specific to particular strains of bacteria, and in the process are themselves regenerated so that they can be grown and subcultured. The autosynthetic activity, however, is not manifested in the absence of the living cells which they attack.

These phenomena are of outstanding importance but their consideration is outside the scope of this book. What concerns us here is to note that just as enzyme actions and the autosynthetic process are applied to the growth and reduplication of the cell when the appropriate structural elements are located in the organism in the right way, so conversely they are applied to its destruction when analogous elements are located in the wrong way.

† A. M. James, unpublished experiments.

XII

CONCLUDING OBSERVATIONS

1. Living cells and the second law of thermodynamics

BACTERIA can build up substances the free energy of which is considerable, and, in particular, they continually reduplicate systems in which the whole organization seems to depend upon a remarkable degree of order. They do not operate, however, otherwise than in conformity with the second law of thermodynamics, and their synthetic reactions are accompanied by oxidative or fermentative changes in the primary substrates, with the result that, on balance, as in all natural processes, free energy runs down.

Most common bacterial cells consume oxygen when it is available and oxidize carbohydrates. In the absence of oxygen, some ferment carbohydrates in a series of reactions which may have certain steps and certain intermediate products in common with the series of changes by which the oxidation occurs. In some cells the occurrence of oxidation tends to inhibit concurrent fermentation. The relative importance of the two kinds of action is, of course, different in the aerobic and in the facultatively and obligatorily anaerobic bacteria. The details of the oxidative mechanisms will not be considered here.†

In most cells oxygen seems to act directly upon a complex catalyst, or system of catalysts, containing iron. The essential chemical function is the change in valency of the metal, but the complex structures with which the iron is associated are presumably necessary to provide the correct spacings and intermolecular forces to facilitate adsorptions and juxtapositions of active groupings. Hydrogen is removed from other participating molecules by the action of dehydrogenases, which consist of a specific protein and a co-enzyme. The intermediate reactions are varied and have been the subject of much investigation and discussion: in them substances such as pyruvic acid and the four carbon dicarboxylic acids seem to play prominent parts.‡

However complex and elusive the details may be, there is no difficulty in principle about understanding how the free energy of

† See *A Symposium on Respiratory Enzymes*, Wisconsin, 1942; Höber, *Physical Chemistry of Cells and Tissues*, 1945 (Section 6 by D. R. Goddard).

‡ A. v. Szent-György, *Perspective in Biochemistry*, 1939, 165, but see also Goddard, loc. cit., *supra*.

one reaction can be made available to assist another. This follows at once from the tendency of complicated chemical transformations to proceed in steps (I. 7). What is interesting is that some degree of respiration (or an equivalent fermentation) must apparently go on even when the cell is not performing any thermodynamically costly feats of synthesis, and that a certain continuous drain of free energy is necessary for the cell merely to remain alive. Even spores may quite possibly depend upon an extremely slow respiration.

A cell which remains alive without growing preserves certain highly active chemical groupings in a state of mobilization, ready, when the necessary raw materials are presented, to synthesize fresh substance by reactions very different from those known in the laboratory. It would be quite in accordance with what is now known of chemical mechanisms in general to suppose that in the cell, so long as it is alive, there are preserved numerous and varied free valencies: in other words, that the cell is, or contains, a macromolecular polyfunctional free radical system. Such a system possesses a degree of stability because the free valencies, which in a homogeneous phase would rapidly saturate one another, are held by the organized cell structure in relatively rigid spatial separation. Their protection in this way would not, however, be absolute, and a slow decay would be inevitable. To counteract this a steady regeneration of free valencies by the participation of the system in chain reactions attended with large free energy decrease would be an effective means. The respiratory reactions would maintain enough free valencies to intervene with success when the appropriate substrates for the reactions of growth and synthesis appeared on the scene.

The following scheme represents a possible relation between the processes of formation, use, and decay of free valency sites in a complex structure.

Formation $\quad \ldots \mathrm{CH} + \mathrm{X} = \ldots \mathrm{C}\text{—} + \mathrm{XH}.$

Use

$$\ldots \mathrm{C}\text{—} + \mathrm{AB} = \ldots \mathrm{CA} + \mathrm{B}$$

$$\ldots \mathrm{CH} + \mathrm{B} = \ldots \mathrm{C}\text{—} + \mathrm{BH}.$$

Decay

$$\text{—}\mathrm{C} \ldots \mathrm{C}\text{—} + \mathrm{MN} = \mathrm{MC} \ldots \mathrm{C}\text{—} + \mathrm{N}$$

$$\mathrm{MC} \ldots \mathrm{C}\text{—} + \mathrm{N} = \mathrm{MC} \ldots \mathrm{CN}.$$

It is unlikely that an indefinite time could elapse without the intrusion of some molecule playing the role of MN, which would cause

a gradual saturation of all the free valencies without leading to useful reactions.

This is not the place to speculate upon the detailed chemistry of possible free radical processes. The derivation of hypothetical schemes is in one sense too difficult and in another too easy. Two further considerations are, however, worth pointing out because they are of a quite general nature. The first is that for polycondensation processes substances capable of easy conversion into biradicals would be of great importance. Suppose, for example, that the following reaction occurs

$$NH_3 + CO_2 = NH:CO + H_2O,$$

and that the molecule NH:CO reacts with a free radical: the result is still a free radical capable of adding further units.

$$X— + NH:CO = X—NH.CO—.$$

The second is that combinations of quite simple radicals can be made to yield the most complex substances. Suppose, for example, an aldehyde becomes hydrated and then loses two hydroxyl groups in succession to activating radicals. The bifunctional methylene derivative produced can combine with NH.CO units to give polypeptide units:

$$RCHO \rightarrow RCH\begin{matrix}OH\\OH\end{matrix} \rightarrow RCH\diagup\!\!\diagdown$$

$$RCH\diagup\!\!\diagdown + NH:CO \rightarrow —CH(R)—NH.CO—$$

Finally a word should be said about the scientific status of such speculations. The value of any individual scheme is very low until there is definite experimental evidence that it contributes to the reactions of a real cell. On the other hand, there is some general significance in the realization (*a*) that such radical schemes are in principle possible, (*b*) that they would explain why cell chemistry is utterly different from ordinary laboratory chemistry, (*c*) that the free radicals required to initiate them would have to be formed in reactions with large free energy decrease such as oxidation, (*d*) that even so, the active radicals can only be preserved from decay by their attachment to more or less rigid structures.

2. Some general characteristics of living matter

The question arises how far the method of approach of which some account has been attempted in this book really proves helpful in

leading to an understanding of the living cell. It is obvious enough that on the technical plane more problems have been raised than solved. Many references have been made to protein patterns, but what precisely these patterns are is not known. The spatial configuration of different enzyme regions has been discussed, but no means of determining it has appeared. The scale effect, according to which a new kind of activity may automatically be set in motion when the cell, or part of it, attains a given size, has been evoked, but the details of its operation have not been uncovered. Analogies with crystal growth seem applicable to the processes of autosynthesis and self-reproduction, and the current ideas of chemical kinetics show how the coupling of reactions might occur in many subtly appropriate ways. Yet what structures do in fact synthesize themselves and what the actual reaction sequences may be remains largely a mystery.

A pessimistic conclusion could pernaps be drawn about the balance sheet: but this would refer to the detailed content, hardly to the general form. Protein patterns undoubtedly exist, the enyzmes in the cell must stand in a definite spatial relation to one another, nothing is exempt from the operation of the scale effect, and the laws which govern the formation of crystals and the transformations of unorganized chemical systems do not lose their validity when they are applied to more complex combinations. And these ideas, even without any detailed development, do help one to understand the general kind of behaviour exhibited by cells.

Some of the more obvious characteristics of living matter are its variability; its adaptability and apparently purposeful activity; its power of reproducing itself, not to give quite exact copies, but with the emergence of individuality among its separate units; its dependence upon its previous history, and the evolution of that history, not in completely closed cycles, but potentially along new and unforeseen paths. There sounds an element of the wayward and intractable in these things, and yet quite crude and elementary studies seem to show that bacteria, nearly the simplest forms of life, possess in a rudimentary way all these characters, and, what is more, for reasons which can be stated in physico-chemical terms.

The phenomena of bacterial adaptation, whether to utilize new substrates or to resist inhibitory drugs, are at first sight a very striking manifestation not only of variability but of purposefulness.

Yet two statements can be made, one with complete assurance and the other with reasonable confidence. The first is that these phenomena are experimentally reproducible and subject to mathematical description. The second is that the adaptations can be intelligibly interpreted as automatic responses to changes in relative reaction velocity which lead to new enzyme balances. Nor does this second statement depend for its validity upon the exactness of any one specific hypothesis.

Since the precise make-up of the cell depends, as follows with considerable probability from studies on adaptation, upon the nature of the medium, and since, under most conditions except those of very carefully controlled experiment, the medium changes as growth proceeds, it seems that few bacterial populations can be free from some degree of heterogeneity. To this factor is added that of the potential irregularity both in the moment and in the mode of division—an irregularity which, if it has not the same cause as the variable delays in phase changes of unorganized matter, probably has an analogous statistical basis. The mode of division is also a continuous function of the changing environment. For these reasons and others, no serial sub-culture of bacteria represents a succession of completely closed cycles, though, with artificially designed procedures, a good enough approximation can be achieved for ordinary experimental purposes.

The normal degree of variability of bacteria, as has been seen, is limited, but profounder changes can occur by rare mutations. These seem to depend upon rearrangements of some key structural patterns and to require a high activation energy or a large local entropy decrease. Once they have occurred they persist and reproduce themselves. Species evolution must depend upon the more drastic changes of this kind, and it is doubtful whether it has ever been witnessed in laboratory experiments. In the laboratory the energy factor can be intensified in various ways, but these are nearly always of a kind which cause death and destruction of the cell before they give rise to mutations so profound as to constitute a change of species. They must co-operate with very rare entropy changes to be effective in the evolutionary way.

Ordinarily, bacteria reproduce their own substance more or less exactly. This statement, however, needs amplification. Individual parts of the cell reproduce copies of themselves with what appears

to be some degree of precision (and on this process the analogy of crystal growth seems to shed a reasonable amount of light), but the proportion in which the different parts are built up is susceptible, it seems, of some latitude.

The cell is dependent upon its own history in two senses. In the one sense it is so because it has no organized existence except in virtue of the organization of that from which it grew, and so on back in what appears, at first sight, to be an infinite regress. In some respects this simplifies the whole problem of biophysics, because it relegates to the background any need to explain how the first organized structures arose. In the other sense the cell depends upon its history because changes in properties such as enzyme balance are the result of its immediate past and determine its immediate future.

Given that various molecular configurations are linked in space in a given way, it is not difficult to see how a certain enzyme activity can occur at point A, giving products which reach point B in just the concentrations and at just the right rate to maintain the required enzyme reactions at this second point, and so to permit the harmonious and co-ordinated growth of the whole structure. Reproduction of this structure, in principle, is easily conceivable. Difficulties only begin to arise when one inquires how so beautifully designed a structure could have come into being.

As long as this question is shelved, one can take the view that experimental biophysics deals with the differential equations of living systems, taking the initial or boundary conditions for granted. As a working principle this carries one some considerable distance, but it is not the whole truth of the matter. It leaves out of account the second way in which cells are dependent upon their own history. This must now receive a brief consideration.

3. Long-range and short-range problems

The Lucretian simile of successive generations handing on the torch of life each to the next like runners in a race is just enough in the sense that fresh torches never seem to flame spontaneously out of the darkness. Cells never constitute themselves from unorganized matter before our eyes. Yet, taking a longer view, the process is not purely repetitive. The precise enzyme balance at any moment depends upon the previous conditions of growth, and in its turn modifies the future growth. Even though, normally, this leads

only to fluctuations about a mean which is stable enough to make species characters quite definite, and even though the fluctuations can be reduced, with proper technique, to a magnitude which is experimentally of negligible significance, nevertheless, it means that in principle, the future of the cell is determined, not by its instantaneous state, but by its whole past.

Over and above the relatively unimportant changes, there are the irreversible changes in key portions of the cell pattern which require large energy and entropy fluctuations. These are too rare to affect the short-range experimentally observable history of a bacterial population, but must play a decisive part in its evolution through long ages. And the probability of the occurrence of one of these profounder transformations will itself be a function of the shorter-range fluctuations.†

For practical purposes, then, it seems expedient to separate the short-range problem from the long-range one. The evolution of a bacterial population may be such that, in strict principle, the same ground is never covered twice, and that the complete description belongs to history rather than to physics. Nevertheless, this secular evolution is normally so slow that experiments of a purely physical kind can be made with entire success, just as the physical properties of a hydrogen-oxygen mixture can be measured in spite of the fact that in the course of centuries the system will have changed into water.

The amplitude of the short-range fluctuations may be quite large when the experimental conditions are not adequately controlled. Experience of this may be disconcerting, and if viewed in the light of a knowledge of the long-range changes, may suggest a pessimism which is really founded on no more than faulty technique.

To take a simple example, if one is ignorant of the way in which the lag of a bacterial culture depends, on the one hand, on the age of the parent and, on the other hand, on the degree of adaptation

† Donnan has argued that, in principle, the evolution of living systems in time is not describable in terms of differential equations, but requires integro-differential equations. Thus, defining the internal state of a system by a parameter, c, for the inanimate world one may write

$$dc/dt = kf(c),$$

where k is a function of external parameters. With a living system one must write

$$dc/dt = k_1 f_1(c_t, t) + \int_{t-\lambda}^{t} k_2 f_2(c, \tau)\, d\tau,$$

where k_1 and k_2 are functions of external parameters. (F. G. Donnan, *Acta Biophysica*, 1936, II. i. 1; 1937, III. i. 43.)

to the medium, a series of determinations will give results appearing chaotic or even wilful. Yet, correctly designed experiments reveal a quantitative and reproducible relation between lag and age, and an intelligible dependence of this lag-age relationship itself upon the adaptive treatment of the culture.

Again, *Bact. coli mutabile* which has once been grown in lactose may be said (with a pardonable exaggeration) never to be the same again, yet hardly in a deeper sense than when one says that hydrogen and oxygen through which a spark has been passed are never the same again. And in any event, one knows that the same change will certainly occur with every new sample of the organism which is allowed to grow for the first time in the presence of the sugar.

For the short-range treatment, then, the behaviour of the cell can quite legitimately be regarded as a physico-chemical problem: in other words, one may attempt to discover the differential equations without seeking to account for the boundary conditions themselves.

In the long-range problem one cannot escape the question of how the organization of the cell came into being. It is a perfectly permissible position to decline the challenge of this problem altogether. There is plenty of valuable work to be done on behaviour, without inquiring into origins. The cause and cure of cancer could be discovered by work which limited its objective in that way. The adoption of such a position would make it possible to combine a purely mechanistic view of cell behaviour with a belief in the miraculous origin of the first organized units.

But if the challenge is not declined, the possible answers to the question of origins fall under the following headings: intervention of a creating mind; the operation of a vital force immanent in nature; chance.

The arguments about chance are too well known to need enlarging upon. On the one hand, it is pointed out that given a long enough time any combination of events, however improbable, will occur. If the result of the combination is to give a self-reproducing system, then the question of origins is answered. On the other hand, the improbability of the required combinations is asserted to be so transcendent that origin by chance is inconceivable. Discussion on this basis becomes rather like a debate on the product of zero and infinity.

It may be remarked that the initiation of most of the processes

which go on in the inanimate world about us depends upon thermodynamic fluctuations of no inconsiderable magnitude. And it may also be said that the probability of the first steps in organization may not, perhaps, be quite so infinitesimal as it appears at a brief glance. The first self-reproducing systems may be comparatively simple, and the complications may be added cumulatively during the course of long ages. Imagine a small crystal growing at the expense of a supersaturated solution. A thermodynamic fluctuation has been needed to bring it above the critical size, and thereafter it grows. An ordered structure arises out of a disordered assemblage, but the total free energy decreases, because the running down of potential energy more than compensates the lowered entropy of the spatial distribution. Here is one of the simplest examples of the creation of order from disorder, paid for by concomitant changes. When the crystal exceeds a certain size it may become unstable, because it is no longer capable of standing the internal strains caused by local temperature fluctuations: it breaks up into a number of smaller crystals, each of which can now repeat the process undergone by the parent. Thus the system is in a very rudimentary way organized and self-reproducing, and it depends upon an increase of one term in the total free energy at the expense of another.

Now, imagine a polycondensation reaction in which a solid polymer grows at the expense of simple units. This can give rise to crystalline structures of high molecular weight, and these can be interpolymers of varied texture. They can, as already pointed out, quite easily possess wholly different chemical characters at two ends. If one growing end of such a structure entered into chemical reactions giving products which could be utilized by the other, there would be a rudimentary organization of another kind.

Once the very simplest kind of self-reproducing, functioning structure develops, it can afford to wait as long as may be necessary for the chance encounter with free radicals or other agents which modify it by the introduction of new side chains or new groups giving yet further possibilities of co-ordinated chemical activity.

What the chemical nature of the simplest possible kind of structure may be is wholly mysterious, yet the available analogies support the idea that it may not have to be of such complexity as the present highly evolved forms of living matter would suggest. These have been selected through long ages as the most efficient and the best

adapted, and the crude early models are now obsolete. The viruses show that what appear to be single substances can possess the autosynthetic property, and no doubt their study will presently reveal the inner nature of this more clearly, but they do not throw light on the problem of origins, since they only reproduce themselves at the expense of material derived from living cells.

After an attempt has been made to assess the credibility of the origin of living matter by chance, the question passes beyond the scope of a scientific essay, but it may be worth while briefly following it to its conclusion.

If one rejects the spontaneous formation of the first units capable of evolving into living organisms, then one must assume a creation. A vital force, which is a special property of living matter, is of no help in explaining how inanimate matter, not subject to it, ever became alive. The conception of the vital force is, in any event, an almost completely sterile one.

The rejection of this kind of vitalism does not, it should be said, lead to a materialistic view of the world as a whole, in which mind is obviously more important than anything else. Of this latter fact one is directly aware from one's own conscious existence, which relates to a kind of reality not even describable in physical terms at all. Not the remotest idea of what the experience of seeing colour is, for example, can be conveyed by any specification of electron transitions, wave-lengths, retinal images, nerve impulses, or chemical changes in brain cells. Yet there is some close functional relation between a certain kind of organization of matter and a concomitant consciousness.

This being certain in man and almost certain in the higher animals, there is little reason to assume other than a continuous decrease in the intensity of the conscious element as the organization becomes simpler. But the function, even if continuous, can be very steep, and the conscious element quite negligible by the time unicellular organisms are reached in the descent of the scale. According to this view, there would be no boundary between living and non-living and no boundary between conscious and unconscious.

In physics the dualisms of matter and energy, time and space, and waves and particles have lost their former supposed sharpness and been replaced by complexes partaking of the nature of both. So, by a distant analogy, may it be with mind and matter. In man the

reciprocal action of mind and matter is so pronounced that only familiarity makes one oblivious of it. A conscious mental resolve to raise one's hand, and a whole series of chemical, electrical, and mechanical changes are set in motion: an electron transition in sodium and, indirectly, a mental experience is caused which could not be described by the most expert mathematical physicist to another except in terms of the mental world. The gulf between the mental and physical worlds seems wholly mysterious, but one is, on closer inspection, not much better off in understanding how an electron transition leads to a light wave, or how the mass of the stars becomes the energy of radiation in space. Functional relations are all that one has to deal with even in the physical problems.

The functional relation relating the physical and the mental is unknown, but one might assume the existence of something of the sort, even though it would have to differ rather profoundly from anything known at present. If, however, it is assumed to exist it must, as already observed, vary rapidly with the degree of organization.

By a sort of extrapolation up the scale it is possible to arrive at the conception of interactions transcending anything we normally experience. This thought takes us to regions quite beyond our present terms of reference.

Extrapolation down the scale is easier, for it seems evident that the conscious element fades rapidly into the background. Whether or not it is absent in principle we cannot tell by experiment, but we can verify that it does not intrude itself into most of the experiments that are made on such simple organisms as bacteria. The validity of such experiments does not depend upon the correctness of any philosophical position, but merely involves the almost certain assumption that a particular factor is of negligible importance in a given range. Few physical experiments do not involve similar assumptions in one way or another.

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PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, OXFORD
BY CHARLES BATEY, PRINTER TO THE UNIVERSITY