FREDERICK GRIFFITH, 1879–1941

Genetic Transformation: a Retrospective Appreciation

First Griffith Memorial Lecture

By W. HAYES

*Medical Research Council, Microbial Genetics Research Unit,
Hammersmith Hospital, London, W.* 12

Exegi monumentum aere perennius (Horace, *Odes*, III, xxx, 1).

(I have completed a monument more lasting than brass.)

On the night of 17 April 1941, almost exactly 25 years ago, Fred Griffith and his colleague, W. M. Scott, were killed by a German bomb during an air raid on London. At the time of his death Griffith was about 60 years old. In an obituary written shortly afterwards it was suggested that a fitting memorial to these two men would be the construction of a new Ministry of Health building more worthy of Griffith and Scott, and of the dedicated and important epidemiological research which they had done within the dilapidated environment of their old laboratory. No one then guessed that Griffith had already built his own memorial 13 years previously when, in 1928, he published in the *Journal of Hygiene* his famous and remarkable paper on the significance of pneumococcal types (Griffith, 1928). This evening, in this first Griffith Memorial Lecture, it is my privilege and intention to try to revive for you the essence of Griffith's most outstanding discovery and, in so far as I can, to present it in perspective against the somewhat sophisticated and mature background of modern molecular genetics to which it gave birth.

Fred Griffith has been described as a shy and reticent man, whose quiet kindly manner, and his devotion to his job, made him a lovable personality to those few who got to know him. Outside his work he found his pleasure in ski-ing and in walking on the Sussex downs where he had built a cottage. Like his elder brother Stanley, who died only a few days before him, he was a medical bacteriologist whose primary and abiding interest, and his life's work, was the epidemiology of infectious disease. He believed that a proper understanding of epidemiological problems could come only from more detailed and discriminating knowledge of infectious bacterial species, and of the nature of bacterial virulence and variation. For a time he worked on the typing of tubercle bacilli with Stanley Griffith, whose published work on this topic extended over many years and was prolific. On the contrary, Fred Griffith's output of scientific papers was, by comparison, remarkable for its paucity. In view of the quality and distinction of what he did publish, however, I think that this must be ascribed to an inate humility and capacity for self-criticism so that he offered to posterity only those products of his research which he judged to be new and important.

I suppose that Griffith would have deemed his most valuable contribution to epidemiology to be the discovery that many serological types exist within group A streptococci; these are the causative organisms of what were, at that time, such

prevalent and lethal human infectious diseases as puerperal fever, erysipelas and scarlet fever, not to mention acute tonsillitis and its complications such as rheumatic fever and middle ear disease—now so admirably controllable by penicillin to which these bacteria do not develop resistance. For us, of course, as for all biologists, Griffith's continuing fame rests on his discovery of the transformation of pneumococcal types. If you were now to ask any microbial geneticist or molecular biologist, who knew nothing of epidemiology, 'What happened in 1928?,' the odds are that he would at once reply, 'Well, for one thing, Griffith discovered transformation'.

The story of this discovery is told in his 1928 paper, which is the only one he wrote on this topic. Pneumococci are divisible into a number of well-defined types according to the serological specificity of the polysaccharide capsule which they possess. At the same time, the virulence of *all* pneumococcal types is determined by their capsulation which protects the invading bacteria from phagocytosis. Among Griffith's most significant discoveries was the observation, which was quite novel at the time, that more or less stable, non-capsulated and avirulent variant strains could be induced by the growth of capsulated pneumococci in the presence of type-specific antiserum. The first half of the 1928 paper concerns the stability of these avirulent variants. Griffith observed that inoculation of mice with large doses of some of these variants very occasionally produced a lethal infection from which virulent capsulated bacteria were recovered. He thought that this reversion to virulence might be due to the fact that the avirulent bacteria had not entirely lost the capacity to synthesize capsular polysaccharide so that, in dense populations such as were injected, a sufficient concentration of polysaccharide might have been present to restore some kind of autocatalytic process which led to normal capsular synthesis.

If this were so, then it should be possible to revert stable non-capsulated strains to virulence by providing them with exogenous capsular material. To test this idea, Griffith inoculated mice subcutaneously with a mixture of small numbers of living avirulent bacteria, and dense suspensions of heat-killed virulent organisms, neither of which yielded virulent capsulated bacteria when injected alone. He found that mice which received the mixtures frequently died from septicaemia and that capsulated virulent organisms could be isolated from their blood. He gave the name 'transformation' to this phenomenon and, in the field of bacterial genetics at least, this name is still used specifically to describe it.

Griffith found that transformation occurred most frequently when the avirulent bacteria originated from the same capsular type as the heat-killed transforming bacteria. However, the main interest of the phenomenon, both at the time and subsequently, centred on the discovery that avirulent pneumococci originating from one capsular type (say, type II) could be permanently transmuted to another type (say, type I or III) corresponding to that of the heat-killed capsulated bacteria with which they were inoculated into mice. For Griffith, as for all medical bacteriologists both then and for many years afterwards, the interest and importance of transformation lay in the light it shed on the nature of virulence and on such epidemiological problems as the stability of serological types and variations in the incidence of type infections. From these points of view the demonstration that both the type and the virulence of well-defined epidemiological varieties of bacteria

could be specifically altered at will, could hardly have been more dramatic. In fact, Griffith appears to have hesitated for some time before publishing his finds (Obituary, 1941) even though, as he says: 'A few years ago the statement that a type I strain could be changed into a type II or a type III would have been received with greater scepticism than at the present day' (Griffith, 1928). This change in attitude was due, at least in part, to his own studies on bacterial variation.

It seems that the interest of type transformation to Griffith was circumscribed by his concern with epidemiology; having clearly demonstrated the phenomenon he appears not to have attempted to analyse it further, and no further references to it appear among his rather scanty subsequent publications. The fact is that the background of biological knowledge at the time would not, in any case, have held out any obvious clues for further experimental study or even for profitable speculation. Nevertheless, it seems strange, in retrospect, that the most striking and important aspect of transformation as we see it now, namely, that it results in an *inheritable* change of character, is neither mentioned nor implied. However, Griffith did attempt, but failed, to demonstrate transformation in the test tube as well as by means of cell-free extracts, but these experiments were not very rigorous ones. When we consider the stringent requirements later shown to be necessary for reproducible *in vitro* transformation in pneumococci, including the exacting condition of 'competence', the failure of these experiments is not surprising (McCarty, Taylor & Avery, 1946). The nearest Griffith got to an explanation of the phenomenon was a suggestion, based on the comparative thermolability of the transforming capacity of certain (type I) heated suspensions, that it might be mediated, not by the capsular polysaccharide itself, but by 'a specific protein structure of the virulent pneumococcus which enables it to manufacture a specific soluble carbohydrate' (Griffith, 1928).

I must now, for the moment, leave Griffith's original discovery in order to trace the developments which followed from it. As we shall see, it proved, had he but known it, to be a delayed-action fuse which, 25 years after its publication, triggered off an explosion of biological knowledge, comparable only to that ignited a century ago by the work of Mendel. Following the demonstration that transformation can occur in the test tube (Dawson & Sia, 1931) and can be mediated by cell-free extracts of capsulated pneumococci (Alloway, 1933), O. T. Avery and his colleagues at the Rockefeller Institute undertook a systematic investigation into the chemical nature of the transforming principle. The answer did not come until 1944, but when it did it was a surprising one, for transforming ability turned out to reside in molecules of pure, highly polymerized deoxyribonucleic acid (DNA) (Avery, MacLeod & McCarty, 1944). In addition to the purely chemical evidence was the fact that the activity of transforming preparations resisted completely the action of the enzyme ribonuclease, which attacks ribonucleic acid (RNA), and of proteolytic enzymes, while being rapidly and specifically destroyed by deoxyribonuclease (McCarty & Avery, 1946). Later purification studies (Hotchkiss, 1952) virtually excluded the possibility that transforming activity could be ascribed to molecules of any other substance contaminating the DNA preparations. Alternatives to the idea that DNA was the agent of transformation had become too bizarre to be acceptable.

However, in 1944 the climate of opinion was not favourable to the idea, which the study of transformation had now made explicit, that the genetic material consisted

of DNA. DNA was known to be associated with protein in nuclei and chromosomes, but only proteins had been shown to possess specificity and were considered to have enough structural complexity to carry the innumerable instructions required to specify all the functions of even the simplest cell. The fuse had ignited the priming charge, but the explosion was yet to come. Meanwhile, progress developed along two main lines. One of these was the expanding search for other systems of transformation which revealed that the phenomenon, far from being restricted to pneumococci and the character of capsulation, occurs in many bacterial genera and species, while DNA preparations can transform with respect to virtually any character in which the donor and recipient population differ and whose inheritance by recipient bacteria can easily be recognized (see review by Ravin, 1961).

A second profitable line of inquiry was the study of transformation from the point of view of an exercise in genetic analysis; that is, the outcome of transformation was interpreted in terms of the transfer of fragments of genetic material from a donor to a recipient bacterium, where pairing and genetic exchange, or crossing-over, occurs with the allelic region of the recipient chromosome. In this way a part, or parts, of the recipient chromosome are replaced by allelic donor fragments and recombinant bacteria are generated. Studies of this kind were initiated by Harriet Ephrussi-Taylor (1951), a colleague of Avery, and led to establishment of the following facts which clearly equated the molecules of transforming DNA with fragments of genetic material.

(1) Transformation is a two-way process so that, for example, not only may pneumococci which have lost the ability to produce capsules be transformed to capsulation, but capsulated bacteria can also be transformed to non-capsulation. The difficulty lies only in demonstrating this reciprocity, since only one of the alternative pairs of characters can usually be selected in the way, for example, that capsulated pneumococci were selected by their virulence in Griffith's experiments.

(2) The transformed character is not just added to the sum of the characters of the recipient bacterium but replaces its corresponding, or allelic, character. This is implicit in the reciprocity of transformation which shows that either allele can express itself. If the transformed character were additional, the same transformants would be obtained irrespective of which parental strain was used as donor.

(3) Certain characters, often of a quite different nature, are found to be linked in transformation; that is, bacteria transformed with respect to one of the donor characters turn out to be simultaneously transformed for the other with a fixed probability much higher than can be ascribed to the chance occurrence of two independent transforming events (Hotchkiss & Marmur, 1954). This means that the determinants of the two characters must have a fixed physical relationship to one another so that, in transformation, they are frequently transferred together on the same molecule of DNA. Such physical relationships between character determinants could only be equated with the linkage of genes on chromosome fragments.

(4) Finally, transformation between two strains which are deficient in the *same* character can often lead to restoration of the character. For example, DNA extracted from one non-capsulated strain of pneumococci may transform another non-capsulated strain to normal capsulation. The genetic explanation here, of course, is that mutational lesions affecting different genes mediating polysaccharide syn-

thesis can be made good by genetic exchange, since the two parental bacteria, between them, possess a complete set of good genes.

At this point it may prove interesting to illustrate some of these genetic features by looking afresh at Griffith's original transformation experiments and re-interpreting them in the light of what we know know of the biochemistry and genetics of capsular polysaccharide synthesis. In general, synthesis of the type-specific polysaccharides is mediated by a series of enzymes determined, in turn, by a set of

Mixture of pneumococci		Transformants observed	Genetic exchanges yielding the observed transformants	Exchanges in positions
Heat-killed	Living			
S II	+ R II	→ S II	A B II 1|2|3| II	1 and 2 1 and 3
S I	+ R II	→ S I	A B I 1|2|3| II	1 and 3 only
S I	+ R II	→ S II	A B I 1|2|3| II	1 and 2 only
R I	+ R II	→ S II	A B I 1|1a|2|3| II	1 and 1a only

Fig. 1. An interpretation of some of Griffith's transformations of pneumococci in genetical terms. These transformations are recorded in tables VII–XIII of his paper (Griffith, 1928). The pneumococcal strains used in each experiment, and the types of the resulting transformants, are shown on the left of the figure; S I and S II indicate capsulated strains of types I and II respectively, while R I and R II are non-capsulated, rough variants (mutants) of these types. The diagrams on the right show, for each experiment, the positions of genetic exchanges (vertical dotted lines) between recipient chromosome (lower longer line) and donor chromosomal fragment (DNA molecule: upper shorter line), which could yield the observed transformants. The chromosomal regions marked *A* are concerned with that part of the pathway of polysaccharide synthesis common to types I and II capsule; those marked *B* determine the type specificity of the polysaccharide, indicated by the I or II. The site of mutation is shown by — ✱ —. The type of transformant depends on the segment of donor fragment which is incorporated into the recipient chromosome by two genetic exchanges. This may be found for any pair of exchanges by tracing along the recipient (lower) chromosome from the left, then up to the donor fragment at the first exchange point and, finally, down again to the recipient chromosome at the second exchange point. See text.

closely linked genes. In the case of a number of pneumococcal types the early steps of the pathway are common to all, so that genetic defects involving them, and leading to failure of capsule production, can be repaired by transforming DNA from another type. On the other hand, the genes determining those later steps in the pathway which confer type-specificity on the polysaccharide, are strict alternatives

which can substitute for one another *en bloc* but cannot participate in mutual repair. It follows that non-capsulated recipients having a mutation blocking the common part of the pathway can be restored to their original type by DNA from donors of a different type; on the contrary, recipients blocked in the specific part of the pathway can be transformed to the donor type only.

On the left of Fig. 1 are shown some of the mixtures of living non-capsulated pneumococci and heat-killed capsulated pneumococci injected into mice by Griffith, and the types of capsulated transformants he observed. It happens that the same non-capsulated strain was used as recipient in all these experiments. Since, as you see, a type II capsule can be restored to this strain by transformation by a type I donor (3rd cross), we may be sure that the mutation leading to non-capsulation involves a gene concerned with that part of the biosynthetic pathway common to both types I and II polysaccharide. The diagrams on the right indicate the positions of genetic exchange between the donor DNA molecules (represented by the shorter upper line of each pair) and the recipient chromosome (the longer lower line). The regions marked '*A*' carry genes which determine biosynthetic steps common to both pathways, the mutation in the recipient being indicated by the cross, while the '*B*' region is concerned with capsular specificity. Note that in transformation, as in other forms of bacterial sexuality, the fragmentary nature of the genetic contribution of the donor demands at least two genetic exchanges, and in any case an even number, to yield a complete recombinant chromosome.

Now let us look at the results. In the first transformation the original capsulation of the recipient can be restored by an exchange in positions 1 and 2 which substitute a functional *A* region for the mutant region, *or* in positions 1 and 3 in which both *A* and *B* donor regions are inherited. In the second transformation the joint inheritance of these *A* and *B* regions is obligatory since not only must the defective *A* region be made good but the ability to synthesize a type I capsule is also conferred. This is, therefore, an example of linked transformation in which at least two, and probably a considerable number of genes, are inherited in a single transformation event. The third transformation, derived from the same mixture as the second, demonstrates the production of different transformant types depending on the position of the second genetic exchange.

Griffith made no comment on the fourth result which must have puzzled him unless he assumed that it was due to a rare reversion. With the advantage of hind-sight, however, we now know that it was very much more likely to have resulted from transformation. Unfortunately we have no way of inferring whether the non-capsulated derivative of type I, here used as donor, was defective in region *A* or *B*, but if we assume mutations in the *A* regions of both strains, then the production of capsulated progeny must have resulted from recombination between mutational sites in the same or two very closely linked genes. Thus Griffith, besides carrying out the first genetic crosses in bacteria, may also, however unwittingly, have recorded the first recombination event at the level of what is now termed the genetic fine structure. In any case the discrimination of transformation analysis is inherently quite refined, the scale being set by the comparative size of the donor DNA molecules involved. In systems where the donor DNA is artificially extracted, the molecules usually have a mean molecular weight of about ten million and are long enough to carry some twenty genes. This is about one-hundredth the length of the whole

bacterial chromosome and corresponds approximately to one hundred-thousandth the total chromosomal DNA of a mouse cell.

The increasing assurance which the chemical and genetic study of transformation gave, that the genetic material, at least of bacteria, consisted of DNA, was paralleled by increasingly detailed chemical and physical investigations into the structure of DNA itself. Among the most significant of these investigations were the X-ray diffraction analyses carried out by M. F. H. Wilkins and his colleagues (Wilkins, Stokes & Wilson, 1953; Franklin & Gosling, 1953).

From chemical analysis DNA was known to be a long polymer, composed of repeating molecules of a pentose sugar, deoxyribose, joined together by phosphate molecules. To each sugar molecule is attached any one of four bases—the two purines, adenine and guanine, which are double-ring structures, and the two single-ring pyrimidines, thymine and cytosine. Each unit, consisting of base, sugar and phosphate molecules, is called a nucleotide so that the DNA polymer is a poly-nucleotide. The X-ray diffraction analyses showed that the polynucleotide chain is in fact arranged as a helix with the bases, which are flat structures, stacked one above the other, and that DNA probably consisted of more than one polynucleotide chain.

Then, early in 1953, just 25 years after the publication of Griffith's discovery came the culmination of this story when Watson & Crick (1953a), by a brilliant synthesis, created a model structure for DNA which appeared to satisfy all the data of chemical and diffraction analysis. Time has confirmed the correctness of this structure, whose elucidation was the main explosion which the discovery of trans-formation, more than any other single event, had first triggered, and whose shock waves still eddy around and disturb the remotest corners of biology.

The elegance and simplicity of this model were too good not to be true, for it at once revealed the nature of those properties of the genetic material which previously had seemed so mysterious; namely, its ability to replicate itself, to carry genetic information, and to undergo inheritable mutation (Watson & Crick, 1953b). The model comprises two intertwined polynucleotide helices held together, not by the usual strong co-valent bonds, but by the weak and easily disrupted forces of hydro-gen bonding between the bases of the opposing strands which look inwards towards one another. From a biological point of view the most important feature of the model is that, for stereochemical reasons, the hydrogen bonding between the bases of the two helices is highly specific. The regularity of the whole structure requires that adenine bonds only to thymine, and guanine only to cytosine, although there is no restriction whatsoever on the sequence of bases along any one chain. Thus the only irregularity which could carry genetic instructions is the sequence of the four bases, or pairs of bases, along the long axis of the molecule, while accurate transfer of the genetic instructions to the next generation is ensured by the specificity of pairing. If the hydrogen bonds break so that the two polynucleotide strands unwind and separate in a pool of nucleotides, the specific bonding of thymine to adenine and of cytosine to guanine, to reproduce the parental sequence of base-pairs, permits the polymerization of two new strands and the formation of two new daughter duplices identical with the original one. Finally, the mystery of mutation is readily explic-able by errors of replication. For example, Watson & Crick (1953b) originally pointed out that the specificity of base pairing in their model depends on the

hydrogen atoms of the bases adopting their most stable positions. However, a rare tautomeric shift in the position of a single hydrogen atom of adenine, for example, allows this base to pair with cytosine instead of with thymine; at the next replication the aberrant hydrogen will have reverted to its usual position. On the other hand, the cytosine which was erroneously introduced opposite adenine now pairs with guanine so that, in one of the daughter double helices, an original A–T base-pair has been replaced by a G–C pair; a letter in the genetic code has been permanently altered.

Similarly, the mutagenic action of base analogues, and of many physical and chemical agents which distort the structure of DNA, may be explained in a logical way. I suppose the most dramatic and brilliant achievement to emerge from elucidation of the structure of DNA, has been the solution of the genetic code during the past year, so that virtually all the particular triplets of bases which specify each of the twenty amino acids, as well as two types of punctuation mark, are known (see Stretton, 1965).

I do not intend to digress further into the more recent revelations of molecular biology, which could hardly be regarded as in direct line of descent from the discovery of transformation, although perhaps derived from it in a very ancestral way. Instead, I would like to conclude this lecture by looking at a few of the ways in which transformation has been, and is being, used as a tool in biological research.

Transformation has a twofold application. In the first place it may be used for recombination analysis, and in a number of organisms it may be the only method available. An example of the kind of information it can provide, as well as an example of the way fragmentary inheritance can be a positive asset in certain kinds of study, is an analysis of the mechanism of penicillin-resistance in pneumococci made 15 years ago by Hotchkiss (1951). He found that DNA extracted from a highly resistant donor strain was unable to transform sensitive recipient bacteria to more than a fraction of the donor degree of resistance. However, if a culture of one of these low-degree-resistance transformants was again exposed to the same DNA preparation, transformants showing a higher degree of resistance could be obtained. By repeating this process, sensitive bacteria could be transformed to the donor degree of resistance by a single preparation of donor DNA but in a series of transformation events, each step of this series leading to only a fractional increase in resistance. This type of step-wise inheritance, which characterizes resistance to the majority of antibiotics, is an expression of the fact that high-degree resistance can only be achieved by the summation of a number of independent mutations, usually in unlinked genes; in transformation these genes are carried on separate DNA molecules so that normally only one is taken up at a time by any particular recipient bacterium. In contrast, high degree resistance to streptomycin, for example, is due to mutation in a single gene which probably controls ribosomal structure, and so can be transferred to sensitive recipients by a single transformation event.

Transformation has also been used to great effect in the genetic analysis of *Bacillus subtilis* which is an organism with two very interesting features. In the first place it produces spores and therefore offers what is probably one of the simplest examples of differentiation which, thanks to transformation, is directly accessible to joint biochemical and genetic analysis. Secondly, replication of the chromosome in this organism has a distinctive feature which makes it very suitable for studying

how chromosome division is regulated—a new cycle of replication, following emergence from the stationary phase or from spores, always begins at the same point and proceeds around (or along) the chromosome in the same direction in all the bacilli of a culture. This interesting and important phenomenon was discovered, and then confirmed, by means of two quite different types of transformation experiment.

If we assume that chromosome replication begins at one end of the chromosome, or at a fixed point on a circular chromosome, and runs at uniform speed towards the other, and is continuous, then in the great majority of bacteria of a randomly
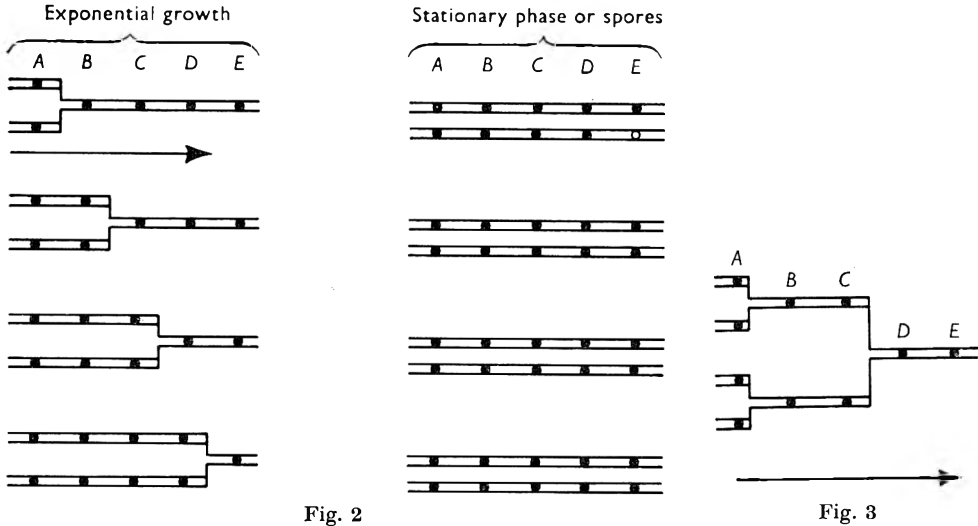
Fig. 2   Fig. 3

Fig. 2. Diagrammatic comparison of the state of chromosome replication among individual *Bacillus subtilis* bacilli in unsynchronized exponentially growing, and stationary-phase populations. The paired lines represent the two polynucleotide strands of the chromosomal DNA. The black circles, designated *A*, *B*, *C*, *D*, *E*, indicate various genes distributed along the chromosome. The arrow indicates the direction of chromosome replication (DNA synthesis), and the fork the position of the growing point of replication. It is assumed that replication begins at a specific point, is polarized and is continuous.

On left: in a randomly growing population during exponential growth, the growing point of replication lies somewhere along the chromosome; in very few bacilli will the chromosome have completed one cycle of replication and not have started another. For every copy of gene *E* there will be two copies of gene *A*, while the number of copies of intermediate genes will lie on a 2:1 gradient in proportion to their distance from *A*.

On right: in a stationary-phase (or spore) population, when growth ceases the current cycle of chromosome replication is completed; a new cycle is initiated only on transfer to a fresh medium.

The figure shows that the ratio of the number of copies of a gene in an exponentially growing population to the number in a stationary-phase population varies from 1·0 close to the initiation point, to 0·5 close to the completion point.

Fig. 3. The diagram explains the relationship between an observed 4:2:1 gene ratio, and initiation of a new replication cycle on the two daughter chromosomes before completion of the initial cycle. The diagram follows upon Fig. 2. See text.

growing population, the growing point at any given moment will lie somewhere between the two extremities, as is shown on the left of Fig. 2 where the black circles, *ABCDE*, represent genes. Very few will have just completed a replication cycle and not yet have started the next. It follows that, in the population, there will be twice

as many copies of a gene located at the starting-point, as at the finishing point. Similarly, the numbers of various intermediate genes should lie on a 2 to 1 gradient depending on the distance of each from the starting point. Clearly the existence of such a gradient could be tested by transformation, on the not unreasonable assumption that the number of transformants with respect to any particular gene is proportional to the number of copies of that gene per unit volume of transforming DNA. However, it happens that different characters may be transformed with very different frequencies for quite other reasons. In order to obtain a true estimate of relative gene numbers, therefore, it is necessary to compare, not the *absolute* numbers of transformants with respect to different genes, but the *ratios* of the transforming capacities of DNA, extracted from exponentially growing cultures on the one hand, and, on the other, from static stationary-phase cultures in which replication of the chromosomes of all the bacteria has been completed so that all the genes are present in equal numbers, as shown on the right of Fig. 2. This ratio has been assessed for 11 genes in *B. subtilis* and the values obtained in fact turn out to lie between 1·0 and 0·5, and to be reproducible (Sueoka & Yoshikawa, 1963). The method thus not only provides evidence for polarized chromosome replication in *B. subtilis*, but also allows the relative locations of the genes along the chromosome to be mapped—an advantage which the fragmentary nature of chromosome transfer normally denies to transformation systems.

A peculiar and unexpected bonus from these studies was the finding that when *Bacillus subtilis* cultures are grown in nutrient broth instead of in a chemically defined medium, the resultant halving of the generation time is accompanied by a change of the 2:1 ratio to a 4:2:1 ratio. As Fig. 3 demonstrates, this seemed to indicate that the chromosome keeps pace with the increased growth rate by initiating a new cycle of replication at the starting point on each of the two daughter chromosomes, at a time when the first cycle is still only half completed. This has since been confirmed (Oishi, Yoshikawa & Sueoka, 1964) and greatly favours the prevalent hypothesis that the pace-maker in the bacterial division cycle is not the nucleus or its equivalent, but the state of the cell membrane which could, of course, be a function of cell mass.

The second type of experiment, which confirmed all the results of the first, illustrates well how transformation can help to establish correlations between physical and genetic data. The donor bacteria are grown up into the stationary phase, or allowed to spore, in a medium rich in the heavy isotopes deuterium and ^{15}N, so that their DNA is denser than normal. In Fig. 4 the two dense strands of the DNA double helix are indicated by the heavy lines. The bacteria, whose chromosomes, as we have seen, are presumptively lined up at the starting-point, are now transferred to a medium containing only light isotopes in which synchronous chromosome replication commences again. As Fig. 4 shows, the newly synthesized DNA has one heavy and one light strand instead of two heavy ones; it is therefore less dense than the parental DNA so that, after extraction of the *total* DNA, the newly synthesized molecular fragments can be cleanly separated from the pre-existing heavy molecules by centrifugation in a density gradient. When this newly synthesized DNA, extracted at intervals throughout the first synchronized generation cycle, is analysed by transformation for the genes it carries, these genes are found to appear in it in a strict and reproducible sequence, indicated by *A*, *B*, *C*, *D*, *E* in the diagram, as

replication of the chromosome proceeds; only at the end of the cycle can the preparation of newly synthesized DNA transform with respect to all the genes (Sueoka & Yoshikawa, 1963; Oishi *et al.* 1964).

Transformation still has a unique and irreplaceable role to play in modern biological research, for it remains the principal method of measuring the biological
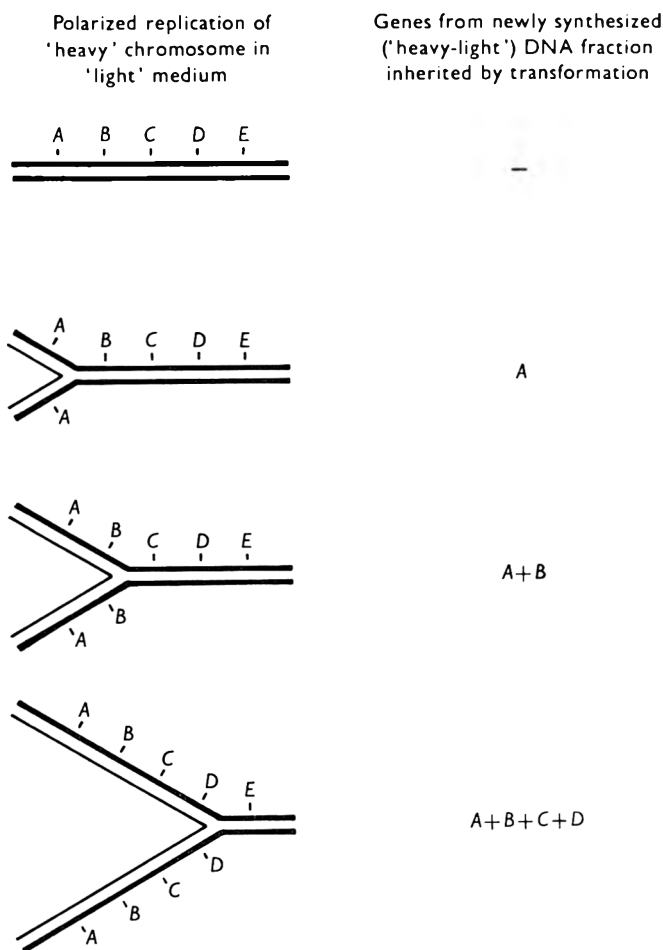

Fig. 4. The diagram illustrates how polarized replication of the chromosome of *Bacillus subtilis* from a specific point may be demonstrated by combined physical and genetic analysis. The heavy lines indicate dense DNA strands which have incorporated ^{2}H and ^{15}N; the light lines are newly synthesized strands of normal density. The diagrams from top to bottom show the progress of DNA synthesis from left to right along the chromosome. The letters, *A–E*, represent a series of genes distributed along the chromosome, whose presence in extracted DNA can be recognized by the transformation of recipient bacilli carrying mutant alleles of these genes. For description of experiment, see text.

activity of DNA. For example, there is little doubt that the ultimate criterion of the *in vitro* synthesis of biologically active DNA from a natural primer will be its transforming ability. Similarly, transformation is a valuable tool in radiation research, or whenever the effects of defined physical or chemical alterations on the biological

activity of DNA must be measured. Thus the phenomenon of photoreactivation, for example (Kelner, 1949), has been found to be due to the action of an enzyme, devoid of species specificity, which combines only with DNA damaged by ultra-violet radiation, requires visible light for its activation, and can restore to u.v.-irradiated transforming DNA a proportion of its biological activity (Rupert, 1961). Without transformation as a meter this enzyme could not have been detected and studied.

In this lecture I have remained loyal to the traditional view of transformation, as a process whereby DNA isolated from a donor strain is able to mediate genetic transfer and recombination between bacteria. But I would like to conclude by extend-ing this concept. The knowledge derived from transformation, that large molecules of nucleic acid, of molecular weight ten million or more, can readily penetrate the walls and semi-permeable membranes of competent bacteria suggested that nucleic acids other than bacterial or, indeed, other than DNA, might similarly gain access to cells. This was first demonstrated for purified ribonucleic acid (RNA) from tobacco mosaic virus which was shown to be infective by itself, though with very low effici-ency as compared with the intact virus equivalent, and to promote the synthesis by the plant of new viral RNA and protein and the release of complete infectious viral particles (Gierer & Schramm, 1956; Fraenkel-Conrat, Singer & Williams, 1957). Since then there have been many examples of the infectivity of nucleic acids, from both plant and animal as well as DNA and RNA viruses. More recently, DNA from a *Bacillus subtilis* bacteriophage has been shown to infect competent transformable bacilli of this organism, with the subsequent liberation of normal phage particles— a process for which the name 'transfection' has been coined (Földes & Trautner, 1964). Thus like transformation, viral infection turns out to be a genetic phenomenon.

A remarkable development of these ideas, stimulated partly by recent experi-mental evidence of the universality of the genetic code, has been the apparently successful attempts to grow animal viruses in transformable bacterial species by exposing competent bacteria to preparations of viral nucleic acids. In this way, complete particles of vaccinia virus have been obtained from *Bacillus subtilis* infected with vaccinia virus DNA, although replication of the viral DNA in the bacteria remains to be proven (Abel & Trautner, 1964). Similarly, by using a special transformation technique, *Escherichia coli* has been infected with RNA from en-cephalomyocarditis virus, with a resulting formation of complete virus particles (Ben-Gurion & Ginsburg-Tietz, 1965). In this case also there is as yet no evidence of replication of the viral RNA, although it is clear that the RNA can behave as a 'messenger' in *E. coli*, determining the synthesis of specific virus protein. Although it is too early to speculate constructively on the future implications of these astonishing experiments, I hope I have said enough to convince you that, in this twenty-fifth anniversary year of Griffith's death, his most important contribution to knowledge remains as topical and controversial as when he discovered it.

REFERENCES

ABEL, P. & TRAUTNER, T. A. (1964). Formation of an animal virus within a bacterium. *Z. Vererbungsl.* **95**, 66.

ALLOWAY, J. L. (1933). Further observations on the use of pneumococcus extracts in effecting transformation of type *in vitro*. *J. exp. Med.* **57**, 265.

AVERY, O. T., MACLEOD, C. M. & McCARTY, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. I. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. exp. Med.* **79**, 137.

BEN-GURION, R. & GINSBURG-TIETZ, Y. (1965). Infection of *Escherichia coli* K12 with RNA of encephalomyocarditis virus. *Biochem. biophys. Res. Commun.* **18**, 226.

DAWSON, M. H. & SIA, R. H. P. (1931). A technique for inducing transformation of pneumococcal types *in vitro*. *J. exp. Med.* **54**, 681.

EPHRUSSI-TAYLOR, H. (1951). Genetic aspects of transformations of pneumococci. *Cold Spring Harb. Symp. quant. Biol.* **16**, 445.

FÖLDES, J. & TRAUTNER, T. A. (1964). Infectious DNA from a newly isolated *B. subtilis* phage. *Z. Vererbungsl.* **95**, 57.

FRAENKEL-CONRAT, H., SINGER, B. & WILLIAMS, R. C. (1957). Infectivity of viral nucleic acid. *Biochim. biophys. Acta* **25**, 87.

FRANKLIN, R. E. & GOSLING, R. G. (1953). Molecular configuration in sodium thymonucleate. *Nature, Lond.* **171**, 740.

GIERER, A. & SCHRAMM, G. (1956). Infectivity of ribonucleic acid from tobacco mosaic virus. *Nature, Lond.* **177**, 702.

GRIFFITH, F. (1928). Significance of pneumococcal types. *J. Hyg., Camb.* **27**, 113.

HOTCHKISS, R. D. (1951). Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. *Cold Spring Harb. Symp. quant. Biol.* **16**, 457.

HOTCHKISS, R. D. (1952). The role of desoxyribonucleates in bacterial transformation. In *Phosphorus Metabolism*. (Ed. by W. D. McELROY & B. GLASS), vol. II, p. 426. Baltimore: Johns Hopkins Press.

HOTCHKISS, R. D. & MARMUR, J. (1954). Double marker transformations as evidence of linked factors in desoxyribonucleate transforming agents. *Proc. natn. Acad. Sci., U.S.A.* **40**, 55.

KELNER, A. (1949). Photoreactivation of ultra-violet irradiated *Escherichia coli* with special reference to the dose-reduction principle and to ultraviolet induced mutations. *J. Bact.* **58**, 511.

McCARTY, M. & AVERY, O. T. (1946). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of desoxyribonuclease on the biological activity of the transforming substance. *J. exp. Med.* **83**, 89.

McCARTY, M., TAYLOR, H. E. & AVERY, O. T. (1946). Biochemical studies of environmental factors essential in transformation of pneumococcal types. *Cold Spring Harb. Symp. quant. Biol.* **11**, 177.

OBITUARY: FRED. GRIFFITH (1941). *Lancet* i, 588.

OISHI, M., YOSHIKAWA, H. & SUEOKA, N. (1964). Synchronous and dichotomous replications of the *Bacillus subtilis* chromosome during spore germination. *Nature, Lond.* **204**, 1069.

RAVIN, A. W. (1961). The genetics of transformation. *Adv. Genet.* **10**, 62.

RUPERT, C. S. (1961). Repair of ultraviolet damage in cellular DNA. *J. cell. comp. Physiol.* **57** (Suppl. 1), 57.

STRETTON, A. O. W. (1965). The genetic code. *Brit. med. Bull.* **21**, 229.

SUEOKA, N. & YOSHIKAWA, H. (1963). Regulation of chromosome replication in *Bacillus subtilis*. *Cold Spring Harb. Symp. quant. Biol.* **28**, 47.

WATSON, J. D. & CRICK, F. H. C. (1953a). The structure of DNA. *Cold Spring Harb. Symp. quant. Biol.* **18**, 123.

WATSON, J. D. & CRICK, F. H. C. (1953b). Genetic implications of the structure of desoxyribonucleic acid. *Nature, Lond.* **171**, 964.

WILKINS, M. F. H., STOKES, A. R. & WILSON, H. R. (1953). Molecular structure of deoxypentose nucleic acids. *Nature, Lond.* **171**, 738.

(Delivered to the Society on 4 April 1966)

The Structure of Phage-Like Objects Associated with Non-Induced Bacteriocinogenic Bacteria

By D. E. BRADLEY and C. ANN DEWAR

Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh 9, Scotland

SUMMARY

Phage-like objects obtained from bacteriocinogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* by the differential centrifugation of culture fluids were examined in the electron microscope. Particles with small heads and contractile tails were found associated with strains of *E. coli*. The three *P. aeruginosa* strains all produced numerous headless contractile tails in broth cultures together with a very few phage-like objects with flexible non-contractile tails. The headless tails possessed cores which appeared both solid and hollow in the electron microscope, indicating the presence of some unidentified substance, perhaps nucleic acid. The only type of particle found associated with *L. monocytogenes* was a large bacteriophage with a complex contractile tail. The structure of these particles is described and discussed, and it is concluded that some of them are the bactericidal principles of spontaneously released bacteriocins.

INTRODUCTION

The bacteriocins are a group of highly specific antibiotics, of which only a few have been studied in detail while a great many have been isolated and classified (for recent review see Reeves, 1965). The individual bacteriocin 'species' are usually referred to by a derivation of the Linnaean specific name of the organism which produces them. For example, the bacteriocins of *Escherichia coli* and *Listeria monocytogenes* are called colicins and monocins respectively. The characteristic physiological property of baeteriocins is their ability to kill a sensitive cell, but not to multiply within it. They are perpetuated by genetic information within the host in the form of plasmids, vegetative particles released in the same way as temperate bacteriophages; they may be spontaneously produced in host cell cultures or may have to be induced.

Comparatively few attempts have been made to study the morphology of bacteriocins with the electron microscope. Kellenberger & Kellenberger (1956) showed phage-like particles associated with a colicin-producing strain of *E. coli*. More recently Sandoval, Reilly & Tandler (1965) and Endo, Ayabe, Amako & Takeya (1965) found that colicinogenic *E. coli* WT15 produced similar particles which were identified as the bacteriolytic principle (Colicin 15). These were small-headed phage-like particles with contractile tails and were apparently identical to the virulent coliphage E1 (Bradley, 1963). Electron microscopy has also shown that the purified pyocin produced by *Pseudomonas aeruginosa* strain R consisted of short rods

identical to head ess tails of contractile bacteriophages (Ishii, Nishi & Egami, 1965); the rods were fonnd both contracted and extended.

A few phage-like particles which are apparently identical to bacteriocins, but which have not been classified as such, have been isolated for several Bacillus species (Seaman, Tarmy & Marmur, 1964; Ionesco, Ryter & Schaeffer, 1964; Stickler, Tucker & Kay, 1965; Bradley, 1965a). These-so-called 'killer' particles were described as defective temperate bacteriophages lacking the ability to multiply intracellularly but able to lyse a sensitive cell. They are thus physiologically identical to bacteriocins. Those studied in greatest detail in the electron microscope were of similar basic morphology to bacteriophages with contractile tails, but had un-usually small heads, only about 300 Å in size. More conventional forms with con-tractile and non-contractile tails were also encountered.

From these observations it seems probable that a substantial number of bacterio-cins are phage-l ke particles or phage components. The present communication describes the results of an electron microscopic examination of these particles associated with bacteriocinogenic strains of *Escherichia coli, Pseudomonas aeruginosa* and *Listeria monocytogenes*.

METHODS

Host bacteria. A number of bacteriocinogenic strains of the species mentioned above were kindly supplied by Dr Y. Hamon, Institut Pasteur, Paris, and these are listed in Table 1. In addition, indicator strains were supplied for bactericidal activity tests. These were *Escherichia coli* K12, *Pseudomonas aeruginosa* P1050, and *Listeria monocytogenes* 23.

Table 1. *Spot tests for bactericidal activity*

Species	Strain	Bacteriocin no.	Spot test of bacteria	Spot test of extract
Escherichia coli	CL10	H	+	+
E. coli	K235	K	+	−
E. coli	167	I	+	−
Pseudomonas aeruginosa	KR35	−	±	−
P. aeruginosa	ST-6	−	+	−
P. aeruginosa	TTC	−	±	−
Listeria monocytogenes	1896x	−	+	+

A + sign denotes a clear area around bacteria or a clear plaque produced by a culture extract. A ± sign denotes a veiled surround or plaque. A − sign denotes no activity.

Preparation of bacterial culture extracts. As has already been stated, bacteriocins or temperate phages are usually present in detectable numbers in cultures of the host bacterium. Suitable extracts should therefore contain sufficient particles for electron microscopy after concentration; accordingly, two simple methods of pre-paration were used.

In the first, double agar layer plates (Adams, 1959) of the bacteria were prepared using Oxoid nutrient broth medium at double strength. After incubation overnight at 37°, 2–3 ml. of neutral 0·1 M-ammonium acetate solution was poured onto the plate, which was allowed to stand for 3–4 hr at room temperature with occasional agitation. The extract obtained was then poured off and centrifuged at 2000–3000 *g*

for 20 min. to remove bacteria. The supernatant was centrifuged at 15,000 g for 3–4 hr to sediment any phage-like particles present. The pellet obtained was resuspended in 0·1 ml. of neutral 0·1 M-ammonium acetate and centrifuged again at 2000–3000 g for 20 min. to remove any remaining bacteria. The resulting supernatant suspension was then ready for electron microscopy.

In the second method, a 10 ml. broth culture of host bacteria was incubated for about 5 hr in a shaker at 37°. Bacteria were sedimented at 2000–3000 g for 20 min. In some cases the culture was shaken with chloroform before centrifugation. The supernatant was then centrifuged at 15,000 g for 3–4 hr and the pellet suspended in ammonium acetate solution as above. The latter method was found most satisfactory.

Spot tests for bacteriocin activity. Both bacteria and concentrated culture extracts were tested by the spot test (Bradley, 1964) for activity against sensitive indicators. This was carried out by placing a loopful of bacteria or extract on the surface of a double agar layer plate of the indicator strain. After incubation, local clearing indicated bacteriocin activity.

Electron microscopy. Negative staining with potassium phosphotungstate (PTA) was used throughout. The suspension in ammonium acetate prepared as above was mixed with an equal volume of neutral 2 % (w/v) PTA solution. A carbon-coated grid was then touched on to the surface of the mixture; all but a thin film of liquid was removed from the grid with the edge of a filter paper. When dry, the specimen was ready for electron microscopy, in this case in a Siemens Elmiskop I.

One of the micrographs was printed by rotation (Markham, Frey & Hills, 1963) as follows. A normal print was accurately centred on a gramophone turntable and fixed with adhesive tape. The turntable was then placed under an enlarger containing the original negative, and the projected image of the object of interest was carefully mated with that on the print. The enlarger was then switched off and a piece of printing paper fixed to the turntable on top of the print already there. The enlarger was then switched on for six periods of time, each equal to one sixth of the total exposure required for a normal print; the turntable of the gramophone was rotated by exactly one-sixth of a revolution between each exposure. On developing, a rotation print was obtained in which the information from each 60° segment of the image was integrated with the rest. It was found that some contrast was usually lost by this procedure, and that it was best to use an extra hard grade of paper.

RESULTS

Spot tests for bacteriocin activity

The results of spot tests of both bacteria and the concentrated culture extracts used for electron microscopy are given in Table 1. Strains producing colicins gave most activity, with clear areas more than 1 mm. wide with sharply defined edges round colonies. Those producing pyocins gave little activity, with a 1 mm. surround of usually veiled appearance and a diffuse edge; the exception to this was *Pseudomonas aeruginosa* strain ST-6. Strains of *Listeria monocytogenes* producing monocins (monocinogenic) formed a narrow (1 mm. or less) area which was clear. It can be seen from Table 1 that only two culture extracts were active against appropriate sensitive indicators; the significance of this will be discussed below.

Electron microscopy of culture extracts

Escherichia coli strain CL10. Extracts of plate cultures contained numerous head-less contracted tails. Better results were obtained from broth culture extracts, however, which contained many phage-like particles with heads. In Pl. 1, fig. 1, an example can be seen to be a conventional contractile particle similar to coliphages $\phi 2$ (Bradley & Kay, 1960) and E1 (Bradley, 1963), a form which is common in nature. The head is 650 Å in size and the tail about 1800 Å long. In Pl. 1, fig. 1, the head is filled with what is presumably deoxyribonucleic acid (DNA), and the sheath is extended. There is a thin base-plate, but no obvious striations on the sheath. Both plate and broth culture extracts contained a very small number of larger particles, one of which is shown in Pl. 1, fig. 2. It is a contractile type with ε. 950 Å head and 2800 Å tail; the one shown has a full head and contracted sheath. Others had empty heads.

Escherichia coli strains K235 *and* 167. There were very few phage-like particles associated with these strains; only one or two could be found in each electron micro-scope grid square. They are illustrated in Pl. 1, figs. 3, 4, and can be seen to be basically similar to the CL10 particles. The heads are about the same size (650 Å) but the tail from strain K235 is rather longer (2000 Å). That from strain 167 is, however, only 1500 Å long, but it has a small collar at the top of the contracted sheath.

Pseudomonas aeruginosa strains KR35 *and* ST-6. Strain KR 35 yielded a few phage-like particles with long non-contractile tails. One with a full head is shown in Pl. 1, fig. 5, and an empty one in Pl. 1, fig. 6. The head is about 700 Å in size and exhibits a six-sided outline; the 1800 Å-long tail has no obvious striations. These resemble the Pseudomonas phage PC described elsewhere (Bradley & Kay, 1960). In addition strain KR 35 contained a small number of headless contracted tails. That shown in Pl. 1, fig. 7, is 1400 Å long; it has a solid core and a contracted sheath with what appear to be fibres attached to it. An extract of strain ST-6 contained no intact phage-like particles but a few headless tails in both a contracted and extended state. In Pl. 1, fig. 8, the contracted tail (length 1400 Å) is hollow. Pl. 1, fig. 9, shows an extended tail, also 1400 Å long, and with marked striations of periodicity of about 37 Å. The tail appears to be hollow for the top 225 Å of its length, but solid for the rest. Both these micrographs also show small discs or perhaps spheres about 120 Å in diamteter. Those at the bottom of Pl. 1, fig. 9, suggest a hexagonal outline.

Pseudomonas aeruginosa strain TTC. Like KR35 and ST-6 this strain had headless tails associated with it, but in much larger numbers. This allowed a rather more detailed study. A group of particles is shown in Pl. 2, fig. 10. It can be seen that they are identical to those already described. Their average length is also 1400 Å. They seem to be solid except for the top 200 Å; the two contracted tails in Pl. 2, fig. 10, are also solid. There is an isolated core with a suggestion of periodicity on it at the bottom centre of the micrograph; this is empty and is readily distinguished from the full or solid cores of the contracted tails. More detail is seen in Pl. 3, fig. 11: striations with a periodicity of about 37 Å and the hollow portion of the core, particularly in the bottom right particle. In Pl. 3, fig. 12, the difference between empty and full cores is shown more clearly. The top particle has a full one, and the other two empty ones; this is a particularly important observation which will be discussed below

In a very few cases (Pl. 3, fig. 13) the helical nature of the sheath was visible. It is a coarse helix similar to that found on T-even coliphages (Bradley, 1963). In some cases the core had slipped out of the contracted sheath leaving a short tube (Pl. 3, fig. 14); two are lying end-to-end in this micrograph. The hole of this tube is 90 Å in diameter, but the cores themselves are only 60 Å wide. As suggested from Pl. 3, fig. 12, there is a slight gap between the core and the tube wall of the extended sheath. The diameter of the extended sheath is 180 Å and that of the contracted sheath, 225 Å.

In addition to headless tails, numerous long rod-shaped objects were found (Pl. 3, fig. 15). At first these might be thought to bear no relationship to the particles just described, but their diameter is 225 Å, like the contracted sheaths, and they resemble the polysheath of T-even coliphages (Poglazov & Tickhonenko, 1963; Kellenberger & Boy de la Tour, 1964).

Listeria monocytogenes strain 1896 x. An extract of a broth culture of this strain provided a rich suspension of phage-like particles (Pl. 4, fig. 16). As with *Pseudomonas aeruginosa* these were largely headless tails. However, a few similar tails attached to heads (Pl. 5, figs. 17, 18) were also found and there were numerous tailless heads (Pl. 5, fig. 19) in the preparation. All the heads, whether attached or detached, were empty; they had an average diameter of 1100 Å. The total tail length of 2400 Å can be seen in Pl. 4, fig. 16, to consist of about 1600 Å of sheath with 800 Å of core projecting from the end. The sheath diameter is 200 Å and that of the core about 75 Å. In Pl. 4, fig. 16, there is also a smaller head (600 Å) with a longer core (2700 Å) attached. The sheaths in Pl. 4, fig. 16, have a very fine capsomere structure whose organization is not apparent, though four parallel axial lines are very obvious. The arrangement of the tail tip is clearer in Pl. 5, figs. 17, 18. There is a distinct base-plate with two prongs at its edge (these are shown as fine fibres in Pl. 5, fig. 21) and a wedge-shaped structure below the base-plate. The cores in the first two particles (Pl. 5, figs. 17, 18) are solid, but in the headless ones they are hollow. The appearance of the sheath is also different on particles with heads: neither the fine subunits nor the longitudinal lines can be seen, though the negative staining and preservation was equally good.

In Pl. 5, fig. 20, the base-plate with its fibres is partially detached from a coreless sheath. This preparation also contained some hexagonal plates (Pl. 5, fig. 22) which can be correlated with the base-plate in Pl. 5, fig. 20. They had obviously become detached from the sheaths. The base-plate in Pl. 5, fig. 22, is clearly of six-fold rotational symmetry and may have some morphological subunits on its rim. The use of the rotational printing procedure (Markham *et al.* 1963) clarifies the whole structure; Pl. 5, fig. 23, has been rotated by 120° between each exposure and Pl. 5, fig. 24, by 60°, i.e. three and six exposures per revolution respectively. There is a progressive improvement in definition in each case clearly showing the shape of the fibres, and also revealing the presence of six subunits slightly to one side of the centre of each edge of the hexagon. Since these are more electron-transparent than the rest of the plate they must consist of a greater vertical thickness of protein.

In Pl. 6, figs. 25, 26, isolated cores are shown in the form of the usual hollow tubes. That in Pl. 6, fig. 25, appears to be pulled out from the sheath; beside it there are five small star-shaped objects. Their origin is shown in Pl. 7, fig. 28, where the sheaths have been partly degraded into individual capsomeres. These minute

objects are hexagonal plates with a subnunit at each apex, giving them a star-like appearance; they measure about 100 Å across.

A tail which has lost its base-plate is shown in Pl. 6, fig. 27; its significance will be discussed below.

DISCUSSION

Phage-like particles from Escherichia coli. All three strains of *E. coli* produced highly active colicins (see Table 1) but only one of the extracts prepared for electron microscopy possessed any killing ability. This strain (CL 10) produced an abundance of phage-like particles, whereas the other two contained barely detectable numbers. Bearing in mind the low centrifugation speed used to sediment the particles it would seem that the CL 10 particles represent colicin H but that those of the other two strains do not represent the bactericidal principle. This is of importance since Sandoval, *et al.* (1965) showed particles associated with *E. coli* WT 15, similar to those of CL 10, which were almost certainly the lytic principle. One deduces from this that some colicins are defective bacteriophages, as suggested by Sandoval *et al.* (1965) for colicin 15, and some are much smaller, not being sedimented by the 15,000 g used in the present investigation. As Reeves (1965) pointed out, colicin CA 42-E_2 has a sedimentation coefficient of only 3·6s meaning that it will be quite different in form and function from colicins 15 and H. The suggestion of Ivanovics (1962) and Fredericq (1963) that colicins are the products of defective lysogeny appears, therefore, to be partly confirmed. It does seem, however, that there may be two completely different types within this group of bacteriocins.

Although there is a basic similarity between the particles of colicin 15 illustrated by Sandoval *et al.* (1965) and those of colicin H shown here, their preparation of colicin 15 contained many empty tail-less heads as well as a substantial number of intact phage-like particles. Since the function of the particles in both cases is now defined, they may be considered physiologically similar to the so-called 'killers' of *Bacillus subtilis* mentioned above (see Introduction).

It is interesting to compare the size of the CL 10 particle with that of coliphage E 1 (Bradley, 1963) which is morphologically similar. The heads are the same size (650 Å) but the tail of colicin CL 10 is 1800 Å long compared with 1150 Å for that of the phage.

Particles from Pseudomonas aeruginosa. The headless tails found associated with pyocinogenic strains of *P. aeruginosa* all looked the same and were identical to the purified pyocin described by Ishii *et al.* (1965). As can be seen from Table 1, the spot tests were not conclusive, but because of the similarity to a properly purified pyocin, it is certain that these headless tails represent the lytic principle.

The pyocins have been well illustrated in order to permit a detailed comparison with the one described by Ishii *et al.* (1965). There is no necessity to go into details since it is abundantly clear that the structure of all four pyocins is identical. The only possible difference is in the length of the particles; those described here are 1400 Å long, and those of Ishii *et al.* (1965) were 1200 Å long. This difference is not considered significant. One or two points, however, have been omitted by these authors.

The most important and significant is the presence of both solid and hollow cores in contracted tails; this indicates unequivocally that something is present in the

tube. The conclusion is supported by the observation that the top 200 Å of extended tails is empty; the rest is presumably filled with some undefined substance. A somewhat analogous observation was made on the virulent pseudomonas phage SLO by Bradley (1965b); here a single empty contractile sheath in the extended state with a 20 Å hole down the centre was found. The obvious and most likely deduction is that the material in the core is a short length of nucleic acid. Also supporting this is the fact that the nucleic acid strand of tailed bacteriophages extends from the head to the tail tip (see Bradley, 1965b). Since all known phages with contractile tails have deoxyribonucleic acid (DNA) as their genetic material, it is reasonable to suppose that the strand within these headless tails is of the same kind. Since the core is capable of being emptied and the tail is of a contractile nature, its contents are doubtless injected into the bacterium when the pyocin adsorbs to the cell surface. This short length of DNA could then misdirect the bacterial metabolism and so cause the death of the cell. It is not contended that this mode of action is anything more than a theory suggested by the presence of a structure within the tail tube. However, such a hypothesis could be confirmed by nucleic acid estimations and perhaps by suitable radioactive tracer experiments.

The nature of the minute disc-like objects in Pl. 1, figs. 8, 9, is not known. These are rather larger than those found by Ishii, *et al.* (1965), which seemed to be segments of cores. With a diameter of 120 Å they are also too small to be segments of the extended sheath. The non-contractile phage-like particles found with KR35 are probably temperate bacteriophages.

Phage-like particles from Listeria monocytogenes. No monocins have hitherto been examined in the electron microscope. In view of the biological activity of the electron microscope preparation, it seems that the particles observed here are the bactericidal principle. While basically of a contractile nature, no particles with obviously extended sheaths could be found. However, the sheaths of the particles in Pl. 5, figs. 17, 18, differ in capsomere structure from the others shown, though the sheath length is the same. The cores are solid, whereas the headless tails have empty ones, suggesting that the former are viable and the latter are not. In this case the head would be naturally empty and would play no part in the killing process. Such a particle could represent an intermediate stage between the colicins and the bacillus 'killers' with their full heads, and the pyocins with their headless tails.

Structurally the particles are of unusual interest mainly because it is possible to resolve the shape of the individual tail capsomeres. Two forms have been tentatively observed for those of conventional phages; short tubes and 'globular' subunits. There is no reason why the star-shaped objects should not represent short tubes seen end-on. Unfortunately there is insufficient information in the electron micrographs to suggest a model for their arrangement, let alone any change in arrangement which may attend sheath contraction. Indeed, there is no evidence to suggest that contraction occurs in the conventional manner; from what one can see, there is merely a change in capsomere packing without a shortening of the sheath. If a morphologically similar virulent bacteriophage for *L. monocytogenes* were found, this point could probably be cleared up; those known are non-contractile (Sword & Pickett, 1961).

The tail without a base-plate shown in Pl. 6, fig. 27, is included because it bears a remarkable resemblance to the rhapidosomes or rod-shaped bodies found by

Correll & Lewin (1964) associated with the gliding organism *Saprospira grandis*. The sheath length of the latter is 1200 Å and that of the monocin is 1500 Å. The total length of each is about the same at 2700 Å. The inference is that the *S. grandis* rhapidosome is a particle arising from a degenerate contractile bacteriophage.

The nature of bacteriocins. While only a few bacteriocins have so far been examined in the electron microscope, it is clear that a knowledge of their structure is valuable in elucidating their true nature. From the results obtained above and those cited in the literature, it can be said of the colicins that two kinds exist: on the one hand there are phage-like particles, and on the other smaller as yet unidentified molecules which presumably act in a different way. With the pyocins the position is different. All four examples so far studied are identical and constitute what is essentially a unique type of bacteriolytic particle. Whether or not all pyocins are the same will only be discovered after an extensive survey. The monocin examined is again different and further study is also required to see whether it is typical of the monocins as a group. Some bacteriocins which have only been mentioned here are those specific to species of the genus *Bacillus*; these are the Bacillus 'killers'. A number of bacteriocins specific to *B. megaterium* (megacins) and *B. cereus* (cerecins) also exist (see Reeves, 1965) and it is obviously important to see whether or not they resemble bacteriophages. Above all it is clear that a wide range of morphological and physiological bacteriolytic particles exist in the bacteriocins, and both the general conception and classification of the group will clearly have to be revised in the light of future work.

REFERENCES

Adams, M. H. (1959). *Bacteriophages.* New York and London: Interscience Publishers.

Bradley, D. E. (1963). The structure of coliphages. *J. gen. Microbiol.* **31**, 435.

Bradley, D. E. (1964). The structure of some bacteriophages associated with male strains of *Escherichia coli. J. gen. Microbiol.* **35**, 471.

Bradley, D. E. (1965a). The isolation and morphology of some new bacteriophages specific for *Bacillus* and *Acetobacter* species. *J. gen. Microbiol.* **41**, 233.

Bradley, D. E. (1965b). The morphology and physiology of bacteriophages as revealed by the electron microscope. *J. R. micr. Soc.* **84**, 257.

Bradley, D. E. & Kay, D. (1960). The fine structure of bacteriophages. *J. gen. Microbiol.* **23**, 553.

Correll, D. L. & Lewin, R. A. (1964). Rod-shaped ribonucleoprotein particles from Saprospira. *Canad. J. Microbiol,* **10**, 63.

Endo, H., Ayabe, K., Amako, K. & Takeya, K. (1965). Inducible phage of *Escherichia coli* 15. *Virology* **25**, 469.

Fredericq, P. (1963). On the nature of colicinogenic factors: a review. *J. theoret. Biol.* **4**, 159.

Ionesco, H., Ryter, A. & Schaeffer, P. (1964). Sur un bactériophage heberge par la souche Marburg de *Bacillus subtilis. Ann. Inst. Pasteur* **107**, 764.

Ishii, S., Nishi, Y. & Egami, F. (1965). The fine structure of a pyocin. *J. mol. Biol.* **13**, 428.

Ivanovics, G. (1962). Bacteriocins and bacteriocin-like substances. *Bact. Rev.* **28**, 108.

KELLENBERGER, E. & BOY DE LA TOUR, E. (1964). On the fine structure of normal and 'polymerised' tail sheath of phage T4. *J. Ultrastructure Res.* 11, 545.

KELLENBERGER, G. & KELLENBERGER, E. (1956). Étude de souches colicinogènes au microscope electronique. *Schweiz. Z. Path. Bakt.* 19, 582.

MARKHAM, R., FREY, S. & HILLS, G. J. (1963). Methods for the enhancement of image detail and accentuation of structure in electron microscopy. *Virology* 20, 88.

POGLAZOV, B. F. & TICKHONENKO, A. S. (1963). A study of T2 phage contractile protein properties. (In Russian). *Biokhimiya* 28, 888.

REEVES, P. (1965). The bacteriocins. *Bact. Rev.* 29, 24.

SANDOVAL, H. K., REILLY, C. H. & TANDLER, B. (1965). Colicin 15: possibly a defective bacteriophage. *Nature, Lond.* 205, 522.

SEAMAN, E., TARMY, E. & MARMUR, J. (1964). Inducible phages of *Bacillus subtilis*. *Biochemistry* 3, 607.

STICKLER, D. J., TUCKER, R. G. & KAY, D. (1965). Bacteriophage-like particles released from *Bacillus subtilis* after induction with hydrogen peroxide. *Virology* 26, 142.

SWORD, C. P. & PICKETT, M. J. (1961). The isolation and characteristics of bacteriophages for *Listeria monocytogenes*. *J. gen. Microbiol.* 25, 241.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Small particle from *Escherichia coli* CL10. × 180,000.

Fig. 2. Large particle from *E. coli* CL 10. × 180,000.

Fig. 3. Particle from *E. coli* K 235. × 180,000.

Fig. 4. Particle from *E. coli* 167. × 300,000.

Figs. 5, 6. Full and empty phage-like particles from *Pseudomonas aeruginosa* KR 35. × 180,000.

Fig. 7. Contracted headless tail from *P. aeruginosa* KR 35. × 300,000.

Figs. 8, 9. Headless tails from *P. aeruginosa* ST-6. × 300,000.

PLATE 2

Fig. 10. Particles from *Pseudomonas aeruginosa* TTC showing contracted and extended sheaths and isolated core. × 200,000.

PLATE 3

Fig. 11. Particles from *Pseudomonas aeruginosa* TTC extended sheaths showing striations and tubular capsomeres. × 275,000.

Fig. 12. Contracted sheaths showing empty and full cores. × 275,000.

Fig. 13. Extended sheath showing coarse helix. × 275,000.

Fig. 14. Isolated contracted sheaths. × 275,000.

Fig. 15. Rod-shaped objects. × 165,000.

PLATE 4

Fig. 16. Particles from *Listeria monocytogenes*. × 200,000.

PLATE 5

Particles from *Listeria monocytogenes*

Figs. 17, 18. Particles with heads. × 280,000.

Fig. 19. Isolated head. × 280,000.

Fig. 20. Base-plate being detached from isolated sheath. × 280,000.

Fig. 21. Particle showing base-plate fibres. × 280,000.

Fig. 22. Base-plate. × 415,000.

Fig. 23. The same, printed by rotation at three exposures per revolution.

Fig. 24. The same, printed by rotation at six exposures per revolution.

10

D. E. BRADLEY and C. A. DEWAR

D. E. BRADLEY AND C. A. DEWAR

Plate 4

16

D. E. BRADLEY AND C. A. DEWAR

D. E. BRADLEY AND C. A. DEWAR

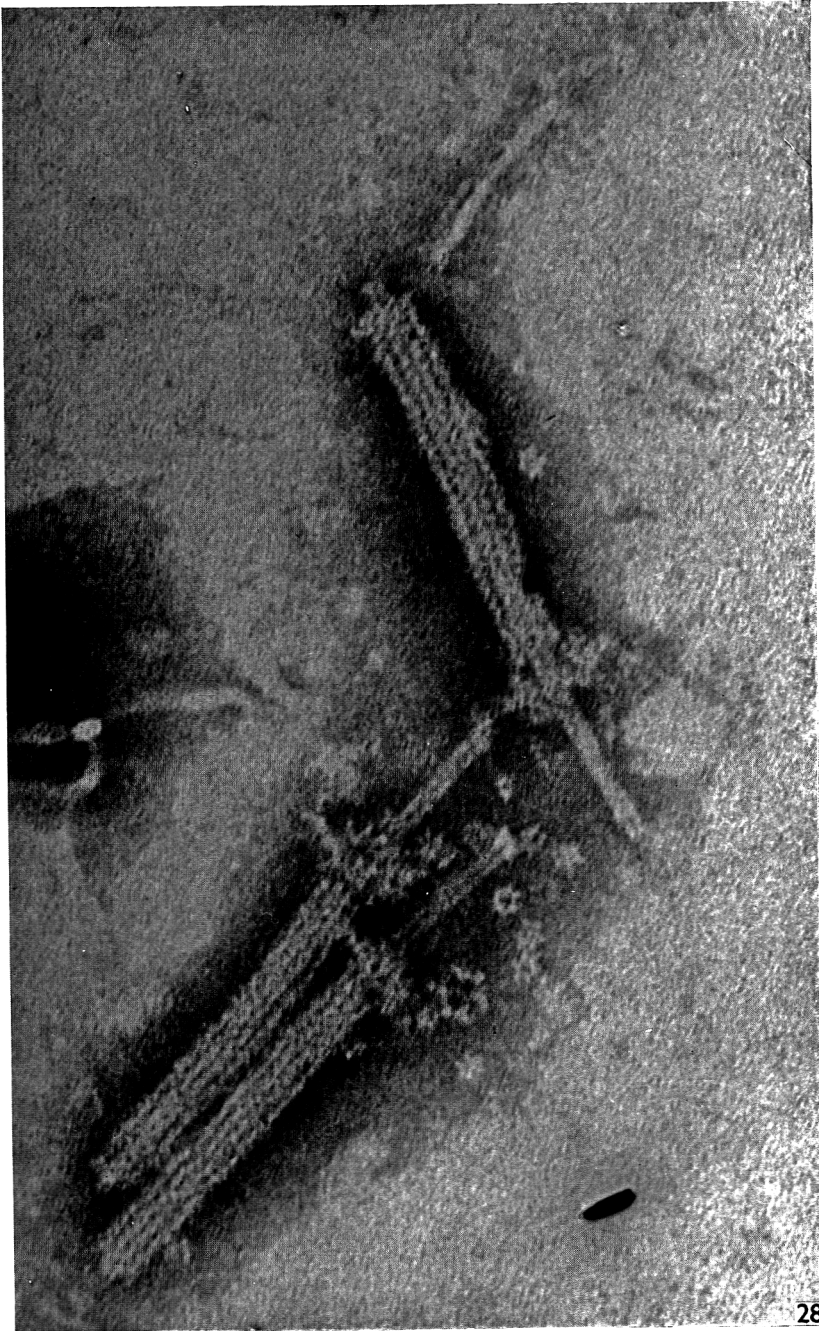D. E. BRADLEY AND C. A. DEWAR

28
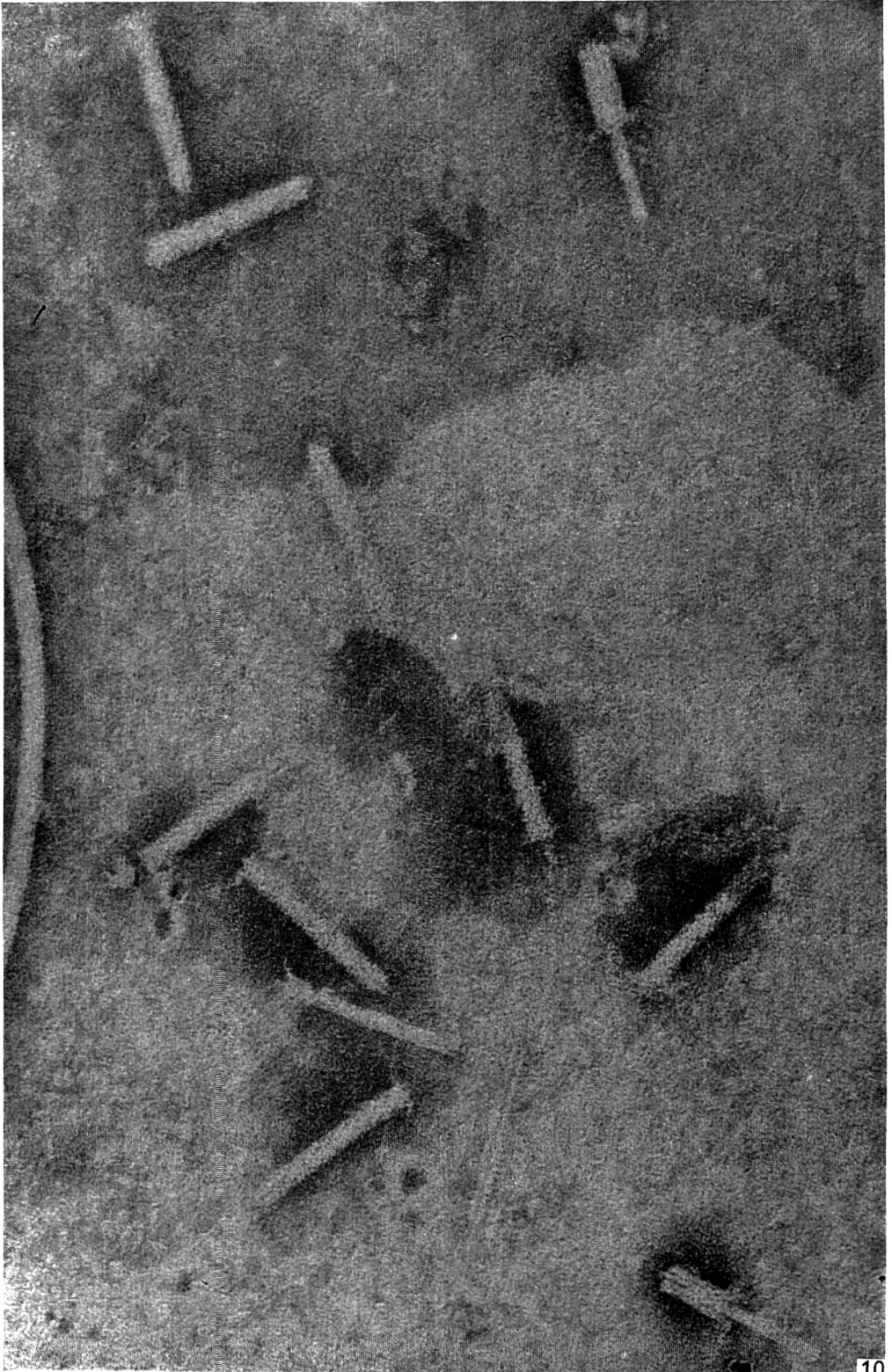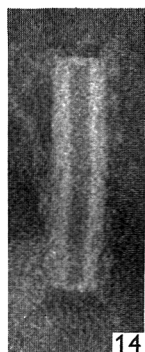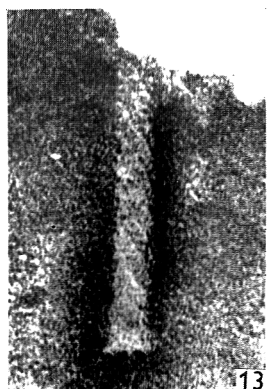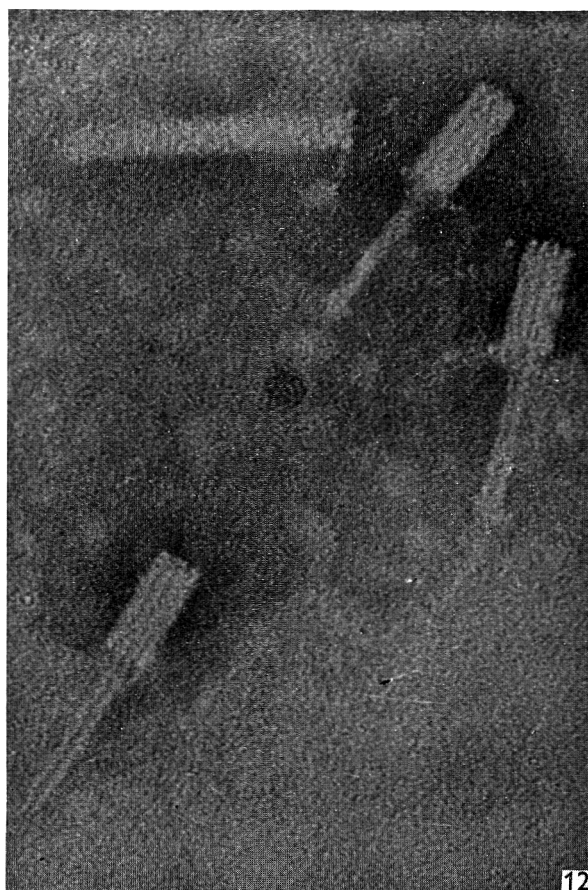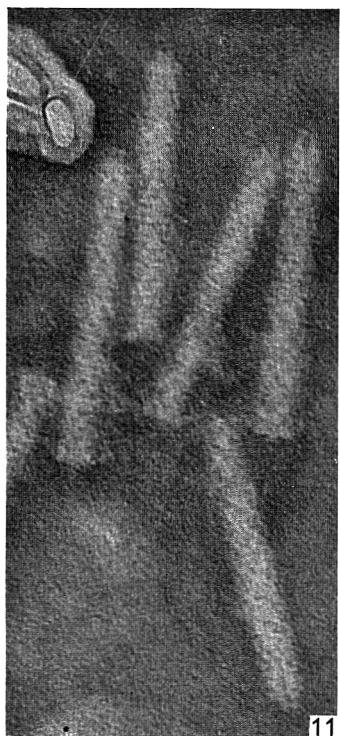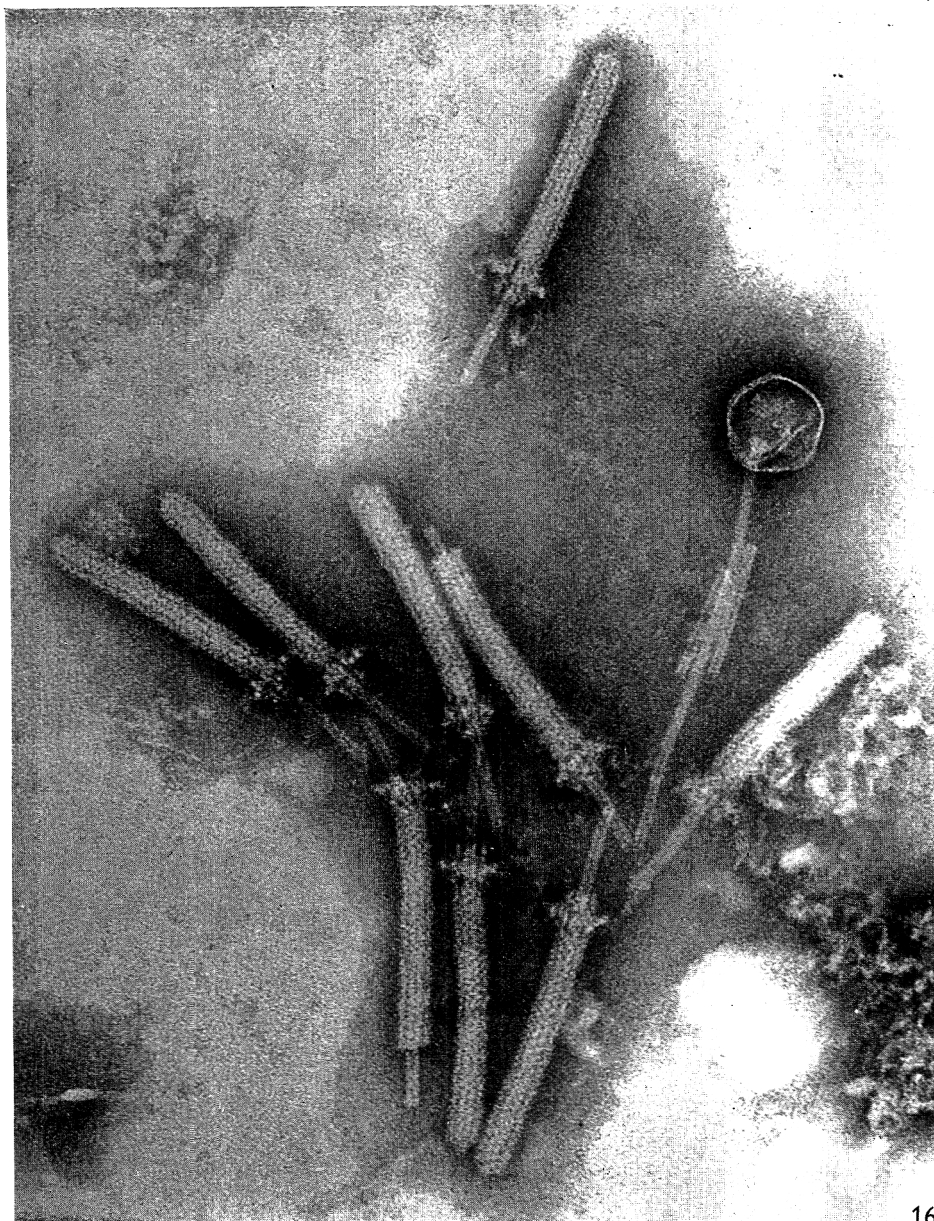
D. E. BRADLEY AND C. A. DEWAR
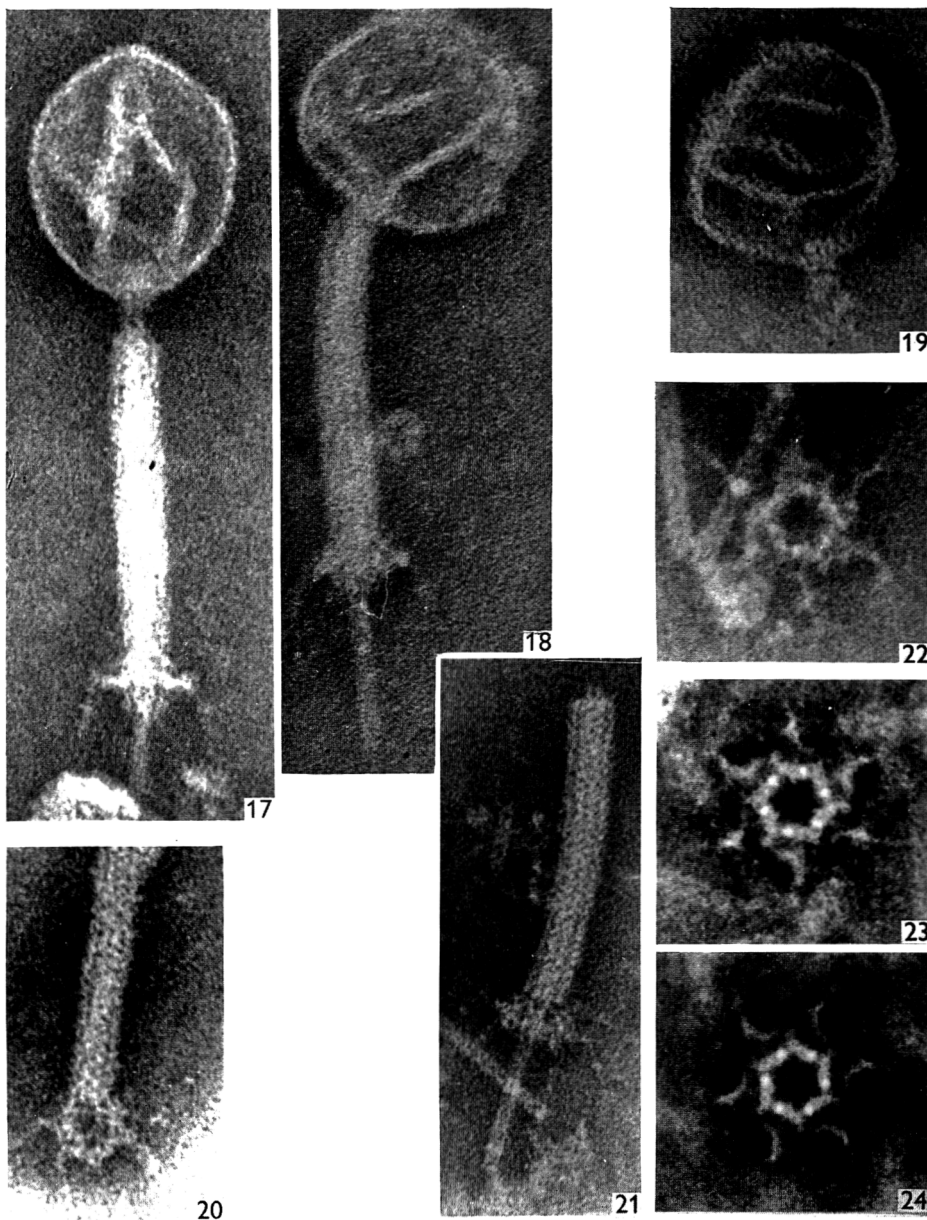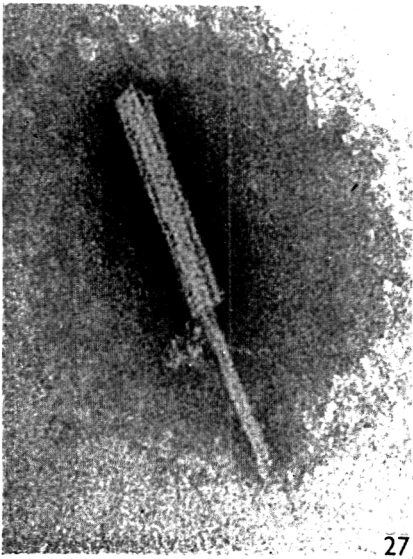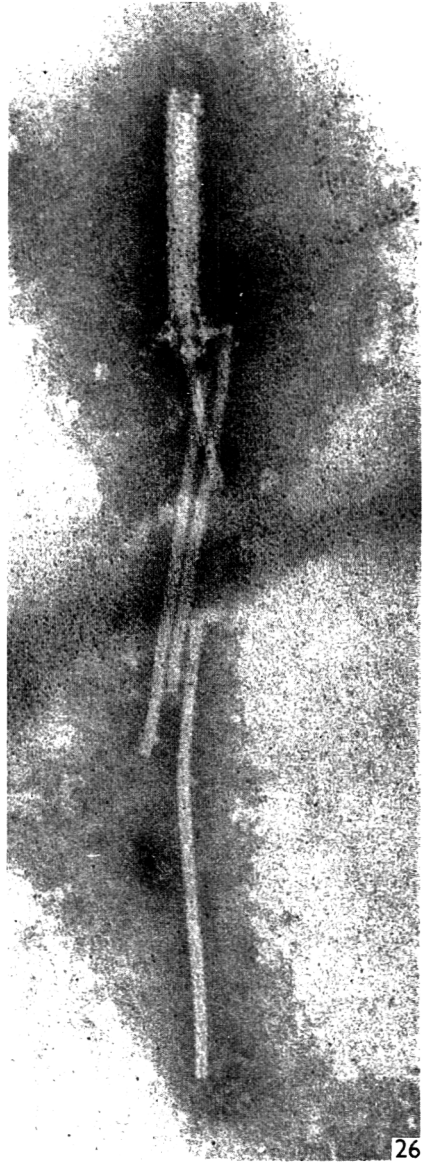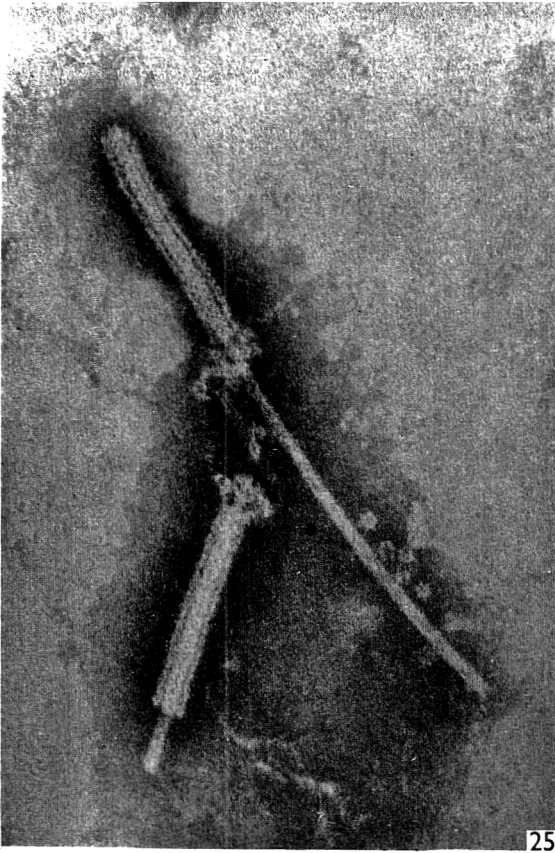
Distribution of Cell Size
in Growing Cultures of Bacteria and the Applicability
of the Collins–Richmond Principle

By A. L. KOCH

*Departments of Biochemistry and Microbiology, College of Medicine,
University of Florida, Gainesville, Florida, U.S.A.*

SUMMARY

Theoretical cell-size distributions for populations of growing cells are calculated for different models of cell growth and for different degrees of variability in size of cells at division. From these computations, it is concluded that the coefficient of variation (c.v.) is almost independent of the relationship of growth rate to cell size. It is 20 % if there is no variability in the cell size at division. For a case typical for enteric rod-shaped bacteria, the variability in cell size at division is about 10 % and the calculated c.v. in cell size of the population in this case increases to 22–23 %. Calculations based on the microscopic observations of others are in the range of 20–25 %. It is proposed that the c.v. of the size distribution serve as a standard in assessing the accuracy of the electronic instruments that size bacteria.

Evidently, only the higher moments of the population cell size distribution contain information bearing on the growth dependence of the organisms on their size. It is pointed out that this means that the Collins–Richmond principle must be applied only to precise and accurate data.

INTRODUCTION

In a growing culture of bacteria, at any instant of time, there is a range of sizes. The measurement of the distribution of these sizes is important because it defines the laws of growth and of cell division which apply to each individual bacterium in the culture. It is evident that in balanced growth the shape of this distribution depends on several factors. First and most important, it depends on the fact that single-celled bacteria grow by binary fission into two daughters, each capable of further division. Secondly, the distribution depends on the kinetics of the growth of a single organism (the growth rate of a bacterium might depend on the time since division, on the mass of the bacterium, or on other physiological events taking place within the bacterium). Thirdly, the distribution depends on the distribution of sizes of those bacteria in the population which are in the act of division. The question is, can we, or how can we, extract information about the second and third factors from the observed size distribution?

This problem is of current interest because of the availability of electronic instruments which measure the size of bacteria in liquid suspension. All the instruments are based on the principle developed by Coulter: a suspension of particles is pumped through a small orifice, and the change in resistance as the particles enter the orifice

is approximately proportional to the volume of the particle; pulses proportional to the resistance changes are sorted and counted electronically. Commercially available equipment (Coulter Electronic, Inc., Hialeah, Florida, U.S.A.) automatically counts the number of particles and writes out their size distribution. Great improvements in accuracy are now possible because of the improved orifice of Kubitschek (1964). In addition, improved electronics have been introduced in the research laboratories of Kubitschek, Marr and Glaser which increase the speed, reliability, accuracy, and ease of obtaining size distributions. At the present time no one can precisely define the accuracy of these various instruments. The second purpose of the present note is to supply guide lines for their utilization.

Theories yielding mass distribution

Several years ago Koch & Schaechter (1962) derived the distribution of cell sizes on the assumption that growth of protoplasm is exponential throughout the cell division cycle, i.e. that the rate of protoplasm synthesis is directly proportional to the amount of protoplasm in the cell. This would be the logical consequence of the assumption that ribosomes are made continuously and, once made, function with constant efficiencies. When this assumption of exponential growth was combined with a second assumption that cells divide precisely when they achieve a certain critical size, the resultant distribution was found to follow an inverse square law. This particular distribution had also been calculated by McLean & Munson (1961). Mathematically the distribution is

$$\theta(m) = \frac{\bar{c}}{m^2}; \quad \left(\tfrac{1}{2}\bar{c} \leqslant m \leqslant \bar{c}\right)$$

and

$$\theta(m) = 0; \quad (m < \tfrac{1}{2}\bar{c}, \ m > \bar{c})$$

(1)

where $\bar{c}$ is the size at division and m is the mass of individual cells.

When the derivation is extended to include variable sizes of cells at division, the following formula is obtained:

$$\theta(m) = \frac{C}{m^2}\left[\int_0^{2m} g(m)\,dm - \int_0^m g(m)\,dm\right].$$

(2)

Here $g(m)$ is the distribution of cell sizes at the instant of cell division.

More recently Powell (1964) discussed this derivation, criticized it, but came to the conclusion that it would be valid if the distribution $g(m)$ were narrow; and, in such a case, the constant C is the harmonic mean of the $g(m)$ distribution. Graphical representation of equations (1) and (2) are given in Fig. 1. In the dotted line in Fig. 1, the points were computed from equations where the $g(m)$ distribution has been assumed to be normal and to have a coefficient of variation of 10%. This is a value typical of the several experimental determinations reported in the literature (Koch & Schaechter, 1962; Schaechter, Williamson, Hood & Koch, 1962). Also shown is the curve for 20% variation.

Probably there are many circumstances in which these distributions apply, so they are presented in different graphical forms in Figs. 2 and 3. In Fig. 2, the cumulant frequency is presented on log probability paper. It is seen that the distribution approximates the log normal distribution over the central 80% of the cells for a 20% coefficient of variation (c.v.) in the size at division, and from the 20
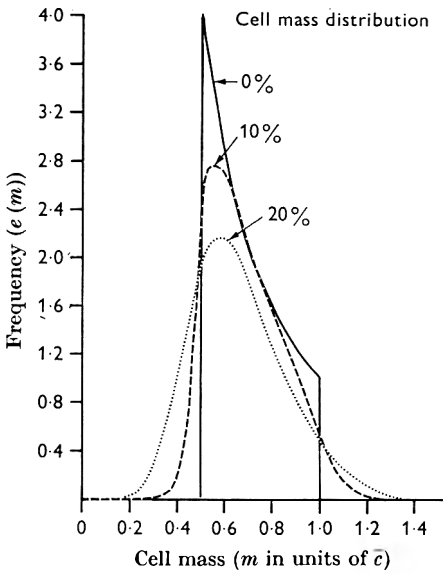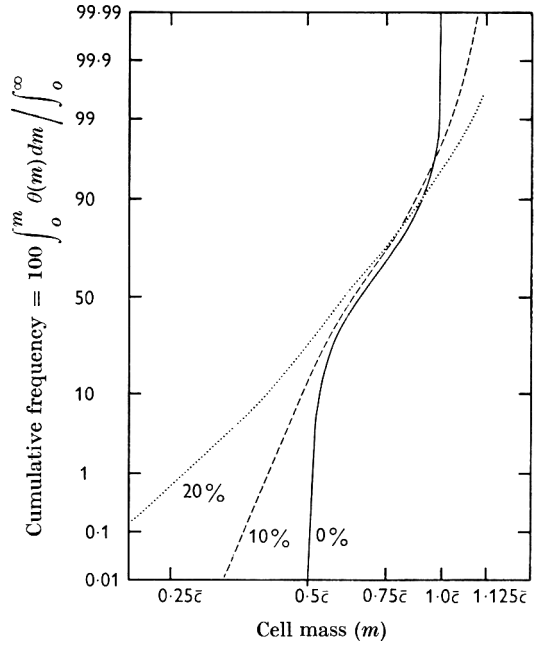
Fig. 1

Fig. 2

Fig. 1. Exponential mass increase model. The distribution of cell sizes for a culture in balanced growth where the mass of each cell increases exponentially at the same rate. Curves are presented for coefficients of variation of the size attained at cell division of 0, 10, and 20 %. The fluctuations are assumed Gaussian.

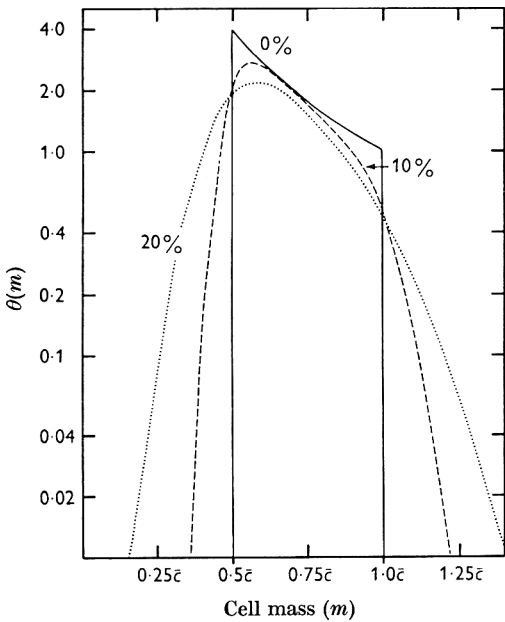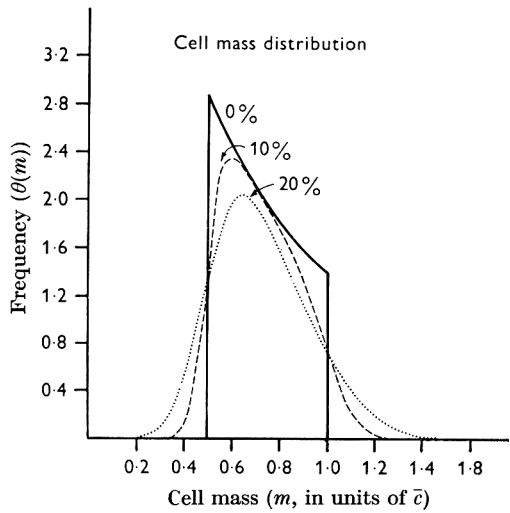Fig. 2. As Fig. 1. but log-probability plot.

Fig. 3

Fig. 4

Fig. 3. As Fig. 1 but in semi-logarithmic representation.

Fig. 4. Linear mass increase model. Other assumptions for the model are given in the legend to Fig. 1 and in the text.

percentile to the 90 percentile for a 10 % c.v. In Fig. 3 the frequency distribution is presented on a semilogarithmic plot. It is presented in this way since this is a convenient representation and because certain of the instruments print out the data this way. Plots of this kind are convenient for estimating the modal size.

Alternatively, it might be assumed that bacterial protoplasm increases at a constant rate, i.e. growth is linear throughout the cell division cycle. A model, although not a likely one, is that only a fully intact genome may serve as template for messenger, and that the level of messenger limits protoplasm synthesis. There are other models that would also predict linear growth. In any case, this is a reasonably extreme hypothesis from the biochemical point of view. On this hypothesis the rate per unit amount of protoplasm doubles when the cell divides into two entities in order that each one of them continues at the same old rate. If it is further assumed that each cell grows from a mass of exactly $1/2\,\bar{c}$ to exactly $\bar{c}$ and then divides, the following distribution is obtained:

and
$$\theta(m) = \frac{8\ln 2}{\bar{c}}\exp\left(-\frac{2m\ln 2}{\bar{c}}\right);\; \left(\tfrac{1}{2}\bar{c} \leqslant m \leqslant \bar{c}\right)$$
$$\theta(m) = 0;\quad (m < \tfrac{1}{2}\bar{c},\, m > \bar{c}). \tag{3}$$

We do not believe this distribution has been presented in the literature before, but its derivation from the well-known age distribution is quite simple and will be omitted. The mean of this distribution was calculated by Cook & James (1964). The distribution is shown in Fig. 4.

The mass distribution for the linear growth case when there is a fluctuation in the size at division is also shown in Fig. 4. The formula can be derived in a manner similar to that used by Powell (1964) and is

$$\theta(m) = C2\ln 2\exp\left(-2\,(\ln 2)m/\bar{c}\right)\int_o^m \frac{\exp\,(2(\ln 2)m/\bar{c})}{m}(g(2m)-g(m))dm, \tag{4}$$

where C is still the harmonic mean of the $g(m)$ distribution. Equation (4) becomes equation (3) as the coefficient of variation of the $g(m)$ distribution decreases.

The Collins–Richmond treatment

Another entirely different approach to this problem was introduced by Collins & Richmond in 1962. These workers derived a relationship between the rate of protoplasm synthesis of cells of a given size in terms of three distributions: the distribution of cell sizes in the balanced growing population, $\lambda(l_x)$; the distribution of cell sizes as the cells divide, $\varnothing(l)$; and the distribution of cell sizes of those cells that are just formed, $\psi(l)$. Their equation, in their symbolism, is

$$V_x = k\left\{2\int_o^{l_x}\psi(e)dl - \int_o^{l_x}\varnothing(e)dl - \int_o^{l_x}\lambda(e)dl\right\}\Big/\lambda(l_x). \tag{5}$$

Here, k is the growth rate constant.

In the derivation of the size distributions given in equations (1)–(4), it has been assumed that cell division divides the cell precisely into equal halves; and so the latter two distributions, ψ and $\varnothing$ of Collins & Richmond, are of precisely the same shape. Equation (2) above can be, and has been, suitably modified when this is not true. Since we have been able to show that division in *Escherichia coli* is quite

precise (Koch, 1966), only the simpler distributions based on this presumption are given here and are used in the calculations given below. With this simplification, we can write the Collins & Richmond equation, in terms of the quantities defined in the present paper, approximately as follows:

$$\theta(m) V_m = 2k \int_o^m \frac{g(2m)\,dm}{m} - k \int_o^m \frac{g(m)\,dm}{m} - k \int_o^m \theta(m)\,dm. \tag{6}$$

This expression is rigorously correct (see note on p. 416) only if the $g(m)$ distribution is very narrow. We will give no computations based on (6), but present it for comparison purposes.

Using this approach, Collins & Richmond calculated that the rate of protoplasm synthesis in *Bacillus cereus* is nearly proportional to the total amount of protoplasm in the individual cell throughout the range of the average cell division cycle, independent of the assumption about the width of the $g(m)$ distribution. Thus, they interpreted their results as being in closer, but not in precise, agreement with the postulate leading to equations (1) and (2) and differing from that leading to equations (3) or (4).

Marr & Harvey (1965) used a modified Coulter counter for the evaluation of the growth law from the Collins–Richmond principle. While they found deviations at extreme sizes, they also concluded that growth is nearly exponential for cells in the normal range of size. While these are highly reasonable conclusions and ones that fit our preconceived notions, it is one of the purposes of the present note to point out that their conclusions require that the cell size distribution be very accurately and precisely shown.

Table 1. *Comparison of exponential and linear growth models for population size distribution*

Coefficient of variation of size at division %	Mean, $\overline{m}$		Coefficient of variation, $s/\overline{m}$		Skewness statistic, γ_1	
	Exp.	Linear	Exp.	Linear	Exp.	Linear
0	$0.6932\overline{c}$(a)	$0.7213\overline{c}$(b)	20.17(c)	19.75 %(d)	+0.4900(e)	+0.2405(f)
5	0.6913	0.7219	20.83	20.40	0.5259	0.2923
10	0.6860	0.7238	22.71	22.09	0.5661	0.3790
15	0.6767	0.7267	25.69	24.62	0.6047	0.4516
20	0.6624	0.7310	29.82	27.68	0.5851	0.4924
25	0.6410	0.7364	35.42	30.96	0.5156	0.4974
30	0.6103	0.7433	42.73	34.66	0.4631	0.4789

$$(a) = \overline{c}\ln 2; \quad (b) = \overline{c}/2 \ln 2; \quad (c) = \sqrt{\left(\frac{1}{2\ln^2 2} - 1\right)} \quad (d) = \sqrt{\left(1 - 2\ln^2 2\right)}$$

$$(e) = (\tfrac{3}{8} - \tfrac{3}{2}\ln 2 + 2\ln^3 2)/(\tfrac{1}{2} - \ln^2 2)^{3/2}; \quad (f) = \left(\frac{1}{4\ln^3 2} - \frac{3}{4}\right)\Big/\left(\frac{1}{4\ln^2 2} - \frac{1}{2}\right)^{3/2}.$$

Statistical parameters of the theoretical distributions

Numerical calculations based on equations (1)–(4) are presented in Table 1. These computations were done on the 709 machine at the University of Florida Computing Center. We have assumed that the distribution function of the sizes at division is Gaussian for these calculations and in those on which Figs. 1–4 are based.

This is an entirely arbitrary presumption. We have also made a judgement as to which of several related yet distinct distributions (see note on p. 416) to take as the normal one (see Powell, 1964, for an exposition). Our basis for the choice is clear and reasonable: as long as we restrict our consideration to relatively small values of c.v. for the size at division, the population size will be relatively insensitive to the shape of the $g(m)$ distribution because of the way in which $g(m)$ enters the calculation. Moreover, the conclusions to be drawn from these computations will be directed solely to the changes in the population size distribution as the type of growth and the dispersion of the distribution of sizes at division are altered.

From the statistical parameters of theoretical distributions given in Table 1, it is seen that it is impossible to deduce anything at all about the growth law by simply measuring the c.v. of the size distribution of cultures in balanced growth. For the case where there is no fluctuation in cell size at division, the c.v. is nearly 20 % for either type of growth. Even if the cell size distribution were very different, the c.v. would not change very much. For example, if the distribution were rectangular from $0 \cdot 5\bar{c}$ to $\bar{c}$, the c.v. would be 19·4 %. Thus, the dispersion of the cell size distribution is largely set by the fact that a cell divides to yield cells that are half as big. The c.v. is significantly different from 20 % only if there are widely dramatic variations in the rate of protoplasm synthesis during division cycle. Such fluctuations are contrary to the limited experimental evidence available (Schaechter *et al.* 1962; Hoffman & Franks, 1965; Cummings, 1965).

Evidently, for its conclusions about the law of protoplasmic synthesis, the Collins–Richmond treatment depends on moments of the distribution higher than the second. Thus, there is a small but significant difference in the third moment about the mean between the two distributions when the size of cells at cell division is precisely determined, but the difference decreases as the fluctuation in the size at cell division is increased.

As fluctuation in the critical size of division is introduced, the measure of skewness, γ_1, at first increases and then decreases. It increases because the third moment about the mean increases as the distribution spreads out at both extremes. The decrease is due to the increase in the standard deviation of the distribution. Since the skewness statistic, γ_1, is defined as the third moment about the mean divided by the cube of the standard deviation, a moderate increase in the standard deviation leads to a decrease in γ_1.

In the absence of experimental bias or error, the standard error of the γ_1 statistic is nearly $\sqrt{(6/n)}$. Thus, 600 cells would be enough to establish a distinction if there were 0 % fluctuation in cell size at division, and 60,000 would be barely enough with 30 % coefficient of variation of division size.

We now return to the most accurately determinable parameter of the distribution of population sizes, namely, its coefficient of variation. It is seen from Table 1 that for either exponential or linear growth, 10 % of fluctuation at division adds only 3 % to the c.v. of the basic distribution, whereas a 20 % fluctuation in size at division adds 8 to 10 % to the basic fluctuation. This leads to the important conclusion that the coefficient of variation of an observed distribution of sizes in balanced growth is a function of the fluctuation of size at division but is almost independent of the kind of growth law.

A CRITERION FOR THE POPULATION SIZE DISTRIBUTION

As far as the author is aware, fluctuation in division size of enteric bacteria at division rarely exceeds 15 % under conditions with normal genotypes in true balanced growth (Schaechter *et al.* 1962), so the coefficient of variation of size distribution should not exceed about 26 %. If a broader cell size distribution than 30 % is found, either experimental artifacts have contributed to broadening the distribution or there are abnormally large fluctuations in the size of the bacteria at division. The resolution between these two possibilities would be necessary before a successful attempt to elucidate the growth law would be possible.

To document this further, we have calculated the c.v. of bacterial size distribution in balanced growth from published and unpublished data available to us (Table 2). Sets of data were used in which various organisms and various experimental techniques were used. All, however, involve visual examination of the bacteria, so that artifacts resulting from the adherence of sister bacteria are presumably not present. In two cases the lengths only were measured and the diameters of the rod-shaped bacteria have been presumed constant. In the other case, both lengths and widths were measured from electron micrographs and volumes computed. We have calculated the coefficients of variation from the workers' published histograms. It is evident that all of these microscopically observed cases fit together with the hypothesis that the critical size at division has a coefficient of variation of not more than 15 %, independent of any detailed hypothesis of the nature of the growth law between the division events.

Table 2. *Coefficient of variation of experimental distributions of
bacterial sizes during balanced growth*

	Type of measurement	Number	Coefficient of variation (%)
Kubitschek & Bendigkeit (1955)			
Escherichia coli B	Volumes	390	25·53
Schaechter (unpublished 1960)			
E. coli B/r	Lengths	206	21·03
Salmonella typhimurium	Lengths	151	20·58
Collins & Richmond (1962)			
Bacillus cereus	Lengths	1932	22·63

THE NEED FOR CAUTION IN INTERPRETING EXPERIMENTAL SIZE DISTRIBUTIONS

The application of the Coulter principle opens new vistas to the study of general physiology, since it can, in principle, accurately define both the control of the division process and the kinetics of protoplasm synthesis at the single cell level. In addition, it can function with particles of the size of bacteria, which it may be hoped will follow simpler laws than may apply to bigger or more differentiated organisms. It can only do this if the instrument allows an accurate estimate of size as well as yielding an accurate estimate of the number of organisms. The latter is achieved simply because a statistically large number of bacteria can be quickly enumerated. The physics of the resistance changes are by no means simple (see, for

example, Gregg & Steidley, 1965). They show that there can be as much as a 50%
difference in the resistance change of particles of the same volume in going from a
spherical shape to a long rod. Evidently, the growth of enteric bacteria by elonga-
tion of the rod at constant diameter could lead to a distortion of the size distribution.
Other difficulties could be mentioned. The most serious one is that alluded to above,
i.e. the possibility that two daughters which have just arisen from the division of
a parent remain attached for a variable length of time and thus artificially broaden
the distribution. Also very serious is the fact, as pointed out by Kubitschek (1964),
that the transit time varies from a minimum for those particles which go down the
aperture axis to very slow passage in the laminar flow near the edges of the orifices.
This can mean that with certain electronic circuits the size of those particles which
go near the axis will be underestimated relative to the others. Other difficulties may
emerge as further work is carried out.

On the other hand, microscopic observations are not without serious drawbacks.
The measurements are very laborious, and it is very difficult to accumulate enough
data on a single population to achieve a highly accurate size distribution. Neces-
sarily, only two dimensions can be measured and the third must be inferred. In some
cases, preparation artifacts due to drying and staining will be introduced. Still,
careful measurements with the optical methods can serve as primary standards to
make sure that the automatic devices are giving a reliable picture.

The proposal outlined above should serve as a check of the overall instrumenta-
tion, if only as a first and crude test. We simply propose that the coefficient of
variation of the size distribution for enteric bacteria in balanced growth under
conditions in which the organisms do not 'snake' should be measured on every
apparatus and under the range of conditions to be used. The results should be a
value consonant with those indicated in Table 1. Then, and only then, should the
effects of drugs or physiological conditions be studied or an analysis based on the
precise shape of the distribution be undertaken. This criterion has been met, at
least for some conditions, in the apparatus of A. G. Marr (personal communication),
and it would be reasonable to expect that such coefficients of variation be control
data to be included with reports about cell size distributions recorded with the
Coulter principle.

NOTE

I have taken $g(m)$ to be the distribution function for the sizes at division. The
o and ψ function in the Collins & Richmond treatment are also distribution functions
for the sizes at division, but relate to a different population of cells. $(g(m))/m$ is
approximately o(m), but $(g(m))/m$ cannot be a true distribution function since its
integral cannot be unity at the same time that the integral of $g(m)$ is unity. Evi-
dently, no error is involved if $g(m)$ is a very narrow distribution. I wish to thank

Dr A. G. Marr very much for pointing this out and for sending me his unpublished calculations based entirely on the Collins–Richmond equation. His calculations do not involve the approximations involved in equations (2) and (4). Comparing his calculated values with ours, it is seen that appreciable error results only when the c.v. of $g(m)$ is greater than 20 %. Even then the error is mainly in the mean value of the distribution and to a much lesser degree in c.v. and γ_1 values.

REFERENCES

COLLINS, J. F. & RICHMOND, M. H. (1962). Rate of growth of *Bacillus cereus* between division. *J. gen. Microbiol.* **28**, 15.

COOK, J. R. & JAMES, T. W. (1964). Age distribution of cells in logarithmically growing cell populations. In *Synchrony in Cell Division and Growth*, p. 405. Ed. by E. Zeuthen. New York: Interscience.

CUMMINGS, D. J. (1965). Macromolecular synthesis during synchronous growth of *Escherichia coli* B/r. *Biochim. biophys. Acta* **95**, 341.

GREGG, E. C. & STEIDLEY, K. D. (1965). Electrical counting and sizing of mammalian cells in suspension. *Biophys. J.* **5**, 393.

HOFFMAN, H. & FRANKS, M. E. (1965). Time-lapse photomicrography of cell growth and division in *Escherichia coli. J. Bact.* **89**, 212.

KOCH, A. L. (1966). On evidence supporting a deterministic process of bacterial growth. *J. gen. Microbiol.* **43**, 1.

KOCH, A. L. & SCHAECHTER, M. (1962). A model for statistics of the cell division process. *J. gen. Microbiol.* **29**, 435.

KUBITSCHEK, H. E. (1964). Apertures for Coulter counters. *Rev. scient. Instrum.* **35**, 1598.

KUBITSCHEK, H. E. & BENDIGKEIT, H. E. (1955). Some studies on the growth and size of bacteria. *Argonne National Laboratories*, **5426**, 99.

MCLEAN, F. I. & MUNSON, R. J. (1961). Some environmental factors affecting the length of *Escherichia coli* organisms in continuous cultures. *J. gen. Microbiol.* **25**, 17.

MARR, A. G. & HARVEY, R. J. (1965). Kinetics of growth of individual cells. *Bact. Proc.* p. 38.

POWELL, E. O. (1964). A note on Koch and Schaechter's hypothesis about growth and fission of bacteria. *J. gen. Microbiol.* **37**, 231.

SCHAECHTER, M., WILLIAMSON, J. P., HOOD, J. R., Jr. & KOCH, A. L. (1962). Growth, cell and nuclear divisions in some bacteria. *J. gen. Microbiol.* **29**, 421.

Heterokaryon Synthesis and Morphogenesis in Verticillium

By J. B. HEALE

Department of Biology, Queen Elizabeth College, (University of London),
Campden Hill, London, W.8

SUMMARY

'Lucerne' auxotrophs of *Verticillium albo-atrum* produced by ultraviolet irradiation and derived from the same parental strain formed heterokaryons on minimal agar 4–5 weeks after inoculation. A degree of heterokaryon incompatibility was shown between auxotrophs derived from different isolates of *V. albo-atrum* and one of *V. dahliae*. Conidial methods of heterokaryon synthesis failed but transference of agar inocula from complete medium agar cultures to minimal medium agar was successful. Conidia were uni-nucleate and nuclear migration subsequent to germ-tube fusion was observed. Heterokaryons grew slowly and were unstable, but by using di-auxotrophs a greater stability was achieved and a number were subcultured successfully for more than a year. Spore analysis yielded colonies of both parental types with various ratios. A heterokaryon test between a hyaline and normal dark auxotroph of *V. albo-atrum* indicated that an important system controlling morphogenesis might be linked with a cytoplasmic factor. A hypothesis is proposed to account for the production and degree of stability of the hyaline variants.

INTRODUCTION

The existence of heterokaryons in laboratory strains of many imperfect fungi, including numerous plant pathogens, has often been shown, although as a natural phenomenon occurring in these organisms its significance has not yet been established (Parmeter, Snyder & Reichle, 1963). By using strains of *Verticillium albo-atrum* from hop Hastie (1962) showed that auxotrophs formed unstable heterokaryons and that as a rare event diploid prototrophic colonies could be isolated from concentrated conidial suspensions. Later (1964) Hastie used strains of *V. albo-atrum* from potatoes and tomatoes to show the probable occurrence of a parasexual cycle. Investigations into the verticillium wilt of lucerne (Heale & Isaac, 1963) showed that isolates of *V. albo-atrum* from this host were virulently pathogenic, whereas *V. dahliae* was only slightly pathogenic. Strains of both fungi from other crops never produced symptoms when inoculated into lucerne, suggesting a marked degree of physiological specialization in the lucerne strains of *V. albo-atrum* and, to a lesser extent, of *V. dahliae*. It was for this reason that the first part of the present work was planned in order to investigate heterokaryon synthesis between 'lucerne' strain auxotrophs and between these and auxotrophs from 'tomato' and 'potato' strains of the fungus.

Apart from its possible significance in leading, with parasexualism, to the recombination of characters controlling virulence and pathogenicity, the ability of

imperfect fungi to form heterokaryons affords a convenient system in which to study morphogenesis. With *Verticillium albo-atrum*, development in culture frequently proceeds along two divergent pathways due to the production of variants or 'saltants'. Wild-type isolates of *V. albo-atrum* are usually characterized by the formation of resting mycelium produced from hyphae which swell, undergo septation and develop darkly pigmented walls. This pigment has been described as a melanin (Brandt, 1962). However, there is no evidence that it is a nitrogen-containing melanin (in the limited sense of Thomas, 1955) and it seems more likely to be a product of catechol oxidation and polymerization (Heale & Isaac, 1964). Laboratory strains of Verticillium invariably produce a hyaline variant which fails to develop resting structures or dark pigment, a phenomenon which has been frequently observed (Pethybridge, 1916; Rudolph, 1931; Beyma Thoe Kingma, 1939; Nelson, 1950; Robinson, Larson & Walker, 1957). Heale & Isaac (1965) showed that the incidence with which such a variant arose was linked with the age of the parent culture and site of inoculum removal; and they suggested that the operative factor might be carried wholly or partly in the conidia.

After a test involving heterokaryon synthesis between a hyaline and normal auxotroph derived from the hop strain of *Verticillium albo-atrum*, Hastie (1962) suggested that cytoplasmic factors played a dominant role in the expression and perhaps inheritance of the mycelial colour. In the present work the possibility of cytoplasmic inheritance of dark resting mycelium was further examined by using a heterokaryon test and auxotrophs derived from the lucerne strain of this fungus. In addition, a single auxotroph of *V. dahliae* characterized by darkly pigmented microsclerotia (distinguished from the resting mycelium of *V. albo-atrum* essentially by the budding process involved in their formation) was used in an attempt to synthesize, and to follow morphological development in, heterokaryons with lucerne, potato and tomato auxotrophs of *V. albo-atrum*.

METHODS

General procedures. Wild-type strains of *Verticillium albo-atrum* Reinke & Berthold were isolated from lucerne and tomato by incubating at 23° small slices of surface-sterilized stem tissue on moistened sterile filter paper. A single strain of *V. dahliae* Klebahn was obtained from lucerne. Single spore isolates were prepared and the strains were grown at 23° in the dark on potato dextrose agar (PDA). Subsequently, except where ability to produce dark resting structures was being checked, a minimal medium agar (MM) was used; it had the following composition (g./l.): sucrose (Analar), 15·0; NaNO₃, 2·0; MgSO₄,7H₂O, 0·5; KCl, 0·5; KH₂PO₄, 1·0; FeSO₄,7H₂O, 0·01; 'Davies' New Zealand agar, 20. Complete medium (CM) was used with MM for the isolation and growth of auxotrophs and contained the constituents of MM plus casein hydrolysate, yeast extract, peptone and malt extract at 1 g./l., and Japanese agar at 2 %.

Production of auxotrophs. Auxotrophs were isolated after ultraviolet (u.v.) irradiation of a water suspension of conidia in an open Petri dish at a distance of 20 cm. from a 2537 Å 'Hanovia' bactericidal tube, model 11 (with ozone reducing filter). Initial tests showed that although the suspension was shaken at an even rate, there was some variation in the irradiation/survival curves obtained. In sub-

sequent work, the suspensions were irradiated at 30 sec. intervals from 1 to 4 min.; plates showing approximately 5 % survival were selected for their yield of mutants. Several methods of total isolation were employed, including master plate and multi-pin replicator, filter paper and velvet pad techniques, the latter being especially well suited for conidial transference in this organism. Di-auxotrophs were similarly produced by further u.v.-irradiation of selected mutants. A list of auxotrophs is shown in Table 1. Three di-auxotrophs originating from a potato strain of *Verticillium albo-atrum* were kindly provided by Dr A. C. Hastie (Botany Department, Queen's College, Dundee), namely 15 (VP 2/7/1) requiring adenine + nicotinic acid, 16 (VP 2/12/7) inositol + tryptophan and 17 (VP2/13/16) uracil + nicotinic acid.

Synthesis of heterokaryons. Three methods of heterokaryon synthesis were attempted:

(*a*) Mixed conidial suspensions were spread on MM agar and incubated at 23° for up to 6 weeks.

(*b*) Mixed conidial suspensions were incubated in test tubes of liquid CM medium for 3 days at 23° until visible growth occurred, followed by streaking on to MM agar and incubation as above.

(*c*) Blocks of agar inoculum containing growing hyphae, conidia and some CM agar, were transferred to MM agar, mixed and incubated for a similar period.

To investigate the initial steps in heterokaryon formation, conidial suspensions were plated on to MM agar and examined after staining with Giemsa or Feulgen; an account of the nuclear condition in *Verticillium albo-atrum* and the methods used will be published elsewhere.

Conidial analysis. Heterokaryotic growths were detected visually and transferred to fresh MM agar. Conidial suspensions were later made with 6-week cultures and were shaken vigorously to ensure complete separation of conidia. From a dilution series on CM agar, either a CM master plate was prepared for multi-pin replication or a sterile velvet pad was used in direct transfer to the appropriate supplemented MM agar.

RESULTS

Auxotrophs

As a result of u.v.-irradiation, germination was markedly delayed in surviving conidia, particularly at the higher dosages. The total yield of auxotrophs was of the order of 1/200 at 5 % survival. Two methionine mutants were lost, presumably due to a high reversion rate; however, strain 2.6, which was utilized later, was more stable with regard to its methionine requirement, probably because of the decreased selection pressure in a di-auxotroph. Strain 1.7 was interesting in that after u.v.-irradiation of the adenine parent, a simultaneous requirement for *p*-amino-benzoic acid (pABA) and pantothenic acid was established. This was difficult to interpret, since pABA is thought to be involved in pantothenate synthesis and one would expect growth on the latter alone in the absence of pABA on the basis of an early block. The specific block in auxotroph 2 was established by adding separately the individual compounds involved in choline biosynthesis; auxotroph 2 appears to be incapable of the initial methylation of ethanolamine. Strain 2.6 characteristically formed dark hyphae but yielded a stable hyaline variant [2.6*] which was used in heterokaryon tests between dark and hyaline auxotrophs.

An orange-coloured mutant (designated M 5) was shown to form a large number of carotenoids absent from the wild type (Valadon & Heale, 1964). In addition, several distinctive morphological strains were produced, including dwarf and wrinkled types, usually with decreased conidiation, and also strains with either loss of or a markedly accelerated synthesis of dark pigment. These mutants have not yet been investigated further.

Table 1. *Auxotrophic strains of Verticillium induced by u.v. irradiation*

Strains 1–11 inclusive were derived from wild-type *V. albo-atrum*; 13 was from an isolate of *V. dahliae*.

Auxotroph	Parent strain	Host	Requirement
1	A	Lucerne	Adeninine
2	A	Lucerne	Choline
4	A	Lucerne	Arginine
1.2	A	Lucerne	Adenine + arginine
1.3	A	Lucerne	Adenine + glycine
1.7	A	Lucerne	Adenine + pABA + pantothenic acid
1.8	A	Lucerne	Adenine + leucine
2.4	A	Lucerne	Choline + arginine
2.6	A	Lucerne	Choline + methionine
11	B	Tomato	Leucine
13	C	Lucerne	Methionine

Heterokaryon formation

Attempts to synthesize heterokaryons using either fresh conidial, or incubated conidial, suspensions of a large number of paired auxotrophs failed.

Using the block technique the following combinations resulted in heterokaryon formation: 1/2, 1/4, 2/4, 1.3/2.4, 1.7/2.4, 1.7/2.6, 1.8/2.4, 1.8/2.6, 2.6*/1.2, 2.6*/1.3 and 2.6*/1.7. All of these were paired lucerne auxotrophs.

With the same technique, the following combinations failed to produce heterokaryons: 1.3/2.6, 11/1.8, 11/2.4, 11/2.6, 11/13, 11/15, 11/16, 11/17, 13/1.8, 13/2.4, 13/2.6, 13/15, 13/16, 13/17, 15/1.3, 15/1.7, 15/1.8, 15/2.6, 15/16, 15/17, 16/2.4, 16/2.6, 16/17. Of these only the 1.3/2.6 combination consisted of paired lucerne auxotrophs.

Heterokaryons were distinguished from auxotrophic and prototrophic growth, due to carry-over and reversion, respectively, by their characteristic morphology and slow growth rate. They were not visible until at least three and in some cases 4–5 weeks after inoculation. After subculture to fresh MM agar, they again grew slowly, usually reaching 30–44 mm. diameter colonies in 6 weeks at 23° as compared with wild-type or supplemented auxotrophic growth of 30–40 mm. colonies in 12 days under similar conditions. The mycelial mats were considerably thicker and usually formed more pale-yellow/greenish pigment (before the production of darkly pigmented resting mycelium) than wild type. In general dark pigmentation was more marked than in auxotrophs growing separately on supplemented MM agar. A further distinction concerned the growth form: heterokaryons showed a greater tendency to zonation and also developed marked irregularities at the edge of the culture.

Heterokaryons derived from single-requirement auxotrophs were not stable on

MM agar and they frequently broke down 2–3 weeks after inoculation on fresh MM agar. In the case of heterokaryon 2/4, 25 hyphal-tip isolations taken from the fine growth developing from the edge of the heterokaryon were found to have an arginine requirement; this indicated that sufficient arginine leakage occurred from the heterokaryon into the surrounding MM agar to allow limited auxotrophic growth to take place. In all cases the original heterokaryons continued to spread across the plate although they were unstable; some of the auxotrophic hyphae produced as a result of breakdown were slowly enveloped, but heterokaryotic growth terminated when the agar medium eventually dried out.

Di-auxotroph heterokaryons were more stable but showed a tendency to break down about 4–5 weeks after inoculation. Despite this instability, several heterokaryons were cultured on MM agar for more than a year (subcultured at 6-week intervals, away from the growing edge of the colony).

The investigation of the nuclear condition of conidia showed them to be regularly uni-nucleate (Pl. 1, fig. 1). Fusion between germinating conidia appeared uncommon, but it was sometimes seen and in one instance nuclear migration through a germ-tube bridge occurred (Pl. 1, fig. 2).

Conidial analysis

Table 2 gives conidial analysis data for six heterokaryons.

Parental types were recovered in each case. The ratio varied from 1:1 to 1:49 and generally the di-auxotrophic heterokaryons were the more unbalanced. A few prototrophic colonies developed when conidia from heterokaryons 1/4, 2/4 and 1/2 were used and these were regarded as revertants. No prototrophic colonies were observed from the di-auxotroph heterokaryons, but only dilute conidial suspensions were used and too few colonies were tested to detect parasexualism.

Table 2. *Verticillium: percentage recovery of parental auxotrophs from six heterokaryons*

Heterokaryon	Requirements of parent auxotroph	Recovery (%)	Requirements of parent auxotroph	Recovery (%)
1/4	Adenine	50	Arginine	50
2/4	Choline	88	Arginine	12
1/2	Choline	36	Adenine	64
1.7/2.6	Choline + methionine	4	Adenine + pABA + pantothenic acid	96
1.3/2.4	Arginine + choline	2	Adenine + glycine	98
2.4/1.7	Arginine + choline	2	Adenine + pABA + pantothenic acid	98

In the test using hyaline and normal auxotrophs all three heterokaryons synthesized, namely 2.6*/1.2, 2.6*/1.3 and 2.6*/1.7 were similar in morphology and growth to those already described. Tests of conidial ratios indicated that whereas these were unbalanced for heterokaryons 2.6*/1.3 (adenine + glycine types predominating) and heterokaryon 2.6*/1.7 (choline + methionine predominating), for heterokaryon 2.6*/1.2 parental types were recovered in a 1:2 ratio; this heterokaryon was selected for further analysis.

Sampling showed that of 98 colonies derived from heterokaryon 2.6*/1.2, 68 required arginine + adenine (1.2) and 30 grew only when supplemented with choline + methionine (2.6). All colonies produced some dark resting mycelium within 2–3 weeks on supplemented MM or on CM agar. At no time before this test did the 2.6* hyaline parent produce any resting mycelium even when incubated up to 6 weeks under these conditions. During subsequent work a small dark area of resting mycelium suddenly appeared in a 5-week culture of this strain on CM agar. Subculture from this plate gave a modified type of 2.6 strain which produced small areas of dark resting mycelium and increased amounts of fluffy white aerial hyphae. Transference to prune lactose agar gave cultures of a similar appearance to that on CM agar. The behaviour of this and other morphological strains carrying biochemical markers is under investigation.

DISCUSSION

The total yield of auxotrophs (0.5 %) was near the expected value, taking into account failures to detect mutants with requirements not present in CM medium and inhibition effects etc. Together with the evidence from stained conidia, these results corroborate the findings of Waggoner (1956), Caroselli (1957) and Hastie (1962) that the conidia of various strains of *Verticillium albo-atrum* are predominantly uni-nucleate. The marked delaying effect of u.v.-irradiation on the growth of surviving conidia is a general phenomenon and is assumed to reflect the operation of repair mechanisms following u.v.-radiation damage.

The colour mutant M5 was found to contain phytoene, β-carotene, γ-carotene, neo-lycopene A, lycopene, neurosporaxanthin and four unidentified pigments (Valadon & Heale, 1964) and it was suggested that their formation resulted from a de-repression leading to the production of a specific precursor in the pathway of pigment synthesis. The results of further work, involving u.v.-irradiation of mutant M5 to produce a range of colour mutants which were then analysed for pigments, were published by Valadon & Heale (1965).

It appears from the present work that lucerne auxotrophs (derived from the same parental strain) will form heterokaryons on MM agar provided that the inoculum is in the form of well-established mycelial growth accompanied by some medium from previous CM agar cultures. Auxotrophic growth is at first predominant under these conditions and heterokaryons are not visible until 4 or 5 weeks after inoculation. This does not imply that heterokaryotic relationships do not become established at an early stage. In fact the evidence from staining germinating conidia shows that fusion of germ-tubes and subsequent migration of nuclei can occur within 6 hr of inoculation. It is interesting in this context to recall the first description of *Verticillium albo-atrum* by Reinke & Berthold (1879), for it included an observation of anastomosing germ-tubes. The present results indicate that something militates against the successful development of a heterokaryon until auxotrophic growth has diminished. In particular, the slow growth rate of the heterokaryon gives it a competitive disadvantage compared with active auxotrophic growth. Furthermore, even when auxotrophic development has more or less terminated, there may still be inhibition of heterokaryotic growth. This was indicated on several occasions by the production of heterokaryons after plating on fresh MM agar of mixed lucerne

auxotrophic (including heterokaryotic initials?) hyphae which had not shown obvious heterokaryotic growth during the previous 6 weeks of incubation.

Since Hastie (1962) successfully used mixed conidial suspensions of the hop strain of *Verticillium albo-atrum* on MM agar to produce heterokaryons, the reasons for their failure to develop when lucerne auxotrophs were used are not clear. Under these conditions, auxotrophic growth is severely limited (particularly in the case of di-auxotrophs) and one would expect selection pressure for prototrophic (hetero-karyotic) growth to be intense. A negative result was also obtained when mixed conidial suspensions were incubated for 3 days in liquid medium CM (when growth was just visible) before plating on MM agar. It may be suggested that the success of the block technique as compared with the two conidial methods was primarily due to the small amount of CM medium transferred with the inoculum, which was sufficient to promote active auxotrophic growth on MM agar. This would lead to multiple hyphal fusions, thereby facilitating heterokaryon synthesis, and at a later stage (3–4 weeks), when auxotrophic growth declined, to visible heterokaryon development.

Only a few auxotrophs were tested in the present work, and since all the hetero-karyons synthesized were derived from a single lucerne strain, it is not possible to conclude that there were significant differences in heterokaryon compatibility between particular host strains of *Verticillium albo-atrum*. It is possible that the relative ease with which the lucerne auxotrophs formed heterokaryons simply reflected the similar genetical constitution of these mutants. However, the results do suggest a heterokaryon incompatibility between auxotrophs derived from dif-ferent isolates; further work is needed to establish whether this is linked with patho-genic or physiological specialization.

The results obtained from heterokaryon analysis with hyaline and normal darkly pigmented auxotrophs suggest that the inheritance of the dark pigment was cyto-plasmically controlled. This is in agreement with the suggestions of Hastie (1962) who worked with hop isolates of the fungus. It is of interest to consider the mechan-ism involved in the production of the hyaline variant. One way in which it might arise is by the failure of a self-replicating cytoplasmic factor to migrate into the young conidium. In a heterokaryon the pigment-synthesizing system would be reconstituted by the normal cytoplasm of the dark parent and, assuming that migration failure is a comparatively rare event, the great majority of conidia from a heterokaryon of this sort would be normal. Brandt & Roth (1965) showed by a dilution technique that a hyaline variant was far less variable than the black parent and that even when u.v.-irradiated it did not produce any black colonies. This supports the suggestion that stable hyalinity is due to the irreversible loss of a cytoplasmic factor which *ipso facto* removes a source of morphological variation. For this hypothesis to be acceptable it must embrace all the known facts of pigment production in Verticillium. In my experience, there are two types of hyaline strains of *Verticillium albo-atrum*. The first is stable, in that dark resting mycelium is probably never formed even under a range of environmental conditions. This is important since light, temperature, nutrient concentrations and age of inoculum have been shown to control the formation of dark resting structures (Heale & Isaac, 1965; Kaiser, 1964; Brandt, 1964). Strains of this type are usually associated with extensive laboratory subculture. The second type, exemplified in the present work

by the 2.6* hyaline strain, apparently retains the latent ability to produce dark pigment. This might be regarded as a reversible change in the cytoplasmic factor rather than an irreversible loss.

Apart from the non-function of an enzyme system which mediates dark-pigment synthesis, the pathways of normal morphogenesis are profoundly altered by the change to hyalinity. In *Verticillium albo-atrum*, resting mycelium (formed by swelling, septation and the development of thickened cell walls in initially slender hyphae) does not develop, considerably more aerial hyphae are produced and spore production is drastically decreased. These widely divergent yet simultaneous changes indicate that an important system controlling morphogenesis in this fungus may be linked to a cytoplasmic factor.

REFERENCES

BEYMA THOE KINGMA, F. H. VAN (1939). Über einige Formen von *Verticillium dahliae* Klebahn. *Antonie van Leeuwenhoek* 6, 33.

BRANDT, W. H. (1962). Manganese stimulation of melanin synthesis and microsclerotia development in Verticillium. *Plant Physiol.* 37 (Suppl.), 30.

BRANDT, W. H. (1964). Morphogenesis in Verticillium: Effects of light and ultra violet radiation on microsclerotia and melanin. *Can. J. Bot.* 42, 1017.

BRANDT, W. H. & ROTH, J. N. (1965). Loss of melanin-containing structures by Verticillium cultures. *Phytopathology* 55, 1200.

CAROSELLI, N. E. (1957). Verticillium wilt of maple. *Bull. R. I. agric. Exp. Sta.* no. 335, 1.

HASTIE, A. C. (1962). Genetic recombination in the hop-wilt fungus *Verticillium albo-atrum*. *J. gen. Microbiol.* 27, 373.

HASTIE, A. C. (1964). The parasexual cycle in *Verticillium albo-atrum*. *Genet. Res.* 5, 305.

HEALE, J. B. & ISAAC, I. (1963). Wilt of lucerne caused by species of Verticillium. IV. Pathogenicity of *V. albo-atrum* and *V. dahliae* to lucerne and other crops; spread and survival of *V. albo-atrum* in soil and in weeds; effect upon lucerne production. *Ann. appl. Biol.* 52, 439.

HEALE, J. B. & ISAAC, I. (1964). Dark pigment formation in *Verticillium albo-atrum*. *Nature, Lond.* 202, 412.

HEALE, J. B. & ISAAC, I. (1965). Environmental factors in the production of dark resting structures in *Verticillium albo-atrum*, *V. dahliae* and *V. tricorpus*. *Trans. Br. mycol. Soc.* 48, 39.

KAISER, W. J. (1964). Effects of light on growth and sporulation of the Verticillium wilt fungus. *Phytopathology* 54, 765.

NELSON, R. (1950). Verticillium wilt of peppermint. *Tech. Bull. Mich. (St. Coll.) agric. Exp. Stn.* no. 221, 1.

PARMETER, J. R. JUN., SNYDER, W. C. & REICHLE, R. E. (1963). Heterokaryosis and variability in plant-pathogenic fungi. *A. Rev. Phytopathology* 1, 51.

PETHYBRIDGE, G. H. (1916). The Verticillium disease of the potato. *Scient. Proc. R. Dubl. Soc.* 15, 63.

REINKE, J. & BERTHOLD, G. (1879). Die Zersetzung der Kartoffel durch Pilze. *Unters. Bot. Lab. Univ. Göttingen.* Berlin: Verl. van Wiegandt, Hempel & Pavey.

ROBINSON, D. B., LARSON, R. H. & WALKER, J. C. (1957). Verticillium wilt of potato. *Res. Bull. agric. Exp. Sta. Univ. Wis.* no. 202, 1.

RUDOLPH, B. A. (1931). Verticillium hadromycosis. *Hilgardia* 5, 197.

THOMAS, M. (1955). Melanins. *Modern Methods of Plant Analysis* 4, 661.

5μ

1

5μ

2

J. B. HEALE
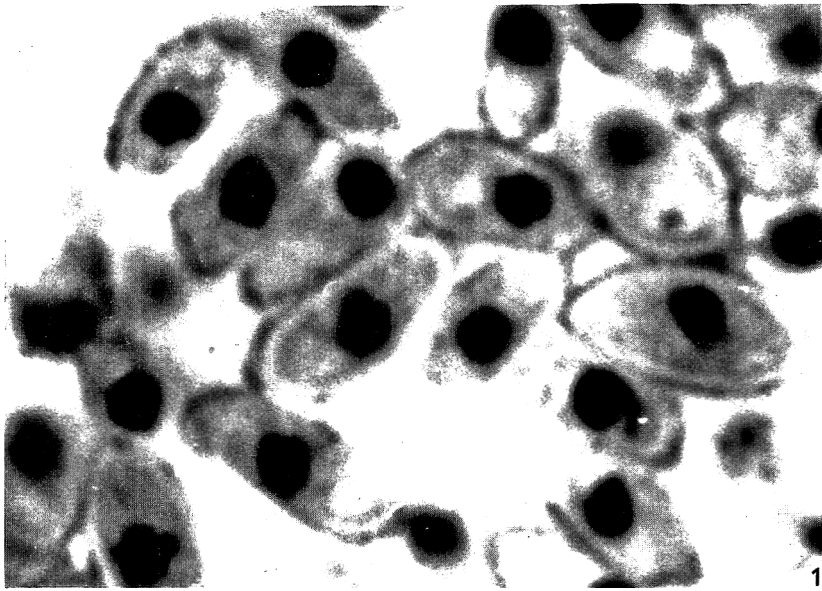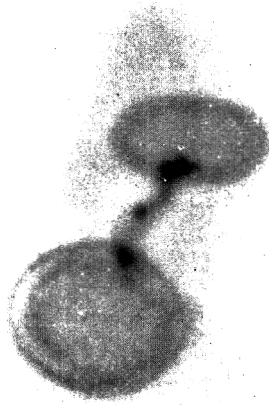
VALADON, L. R. G. & HEALE, J. B. (1964). Carotenoid pigments in a mutant strain of *Verticillium albo-atrum*. Reinke & Berthold. *Nature, Lond.* **202**, 512.

VALADON, L. R. G. & HEALE, J. B. (1965). Carotenoids in mutants of *Verticillium albo-atrum*. *J. gen. Microbiol.* **41**, 341.

WAGGONER, P. E. (1956). Variation in *Verticillium albo-atrum* from potato. *Plant Dis. Reptr.* **40**, 429.

EXPLANATION OF PLATE

Fig. 1. Conidia of a lucerne strain of *Verticillium albo-atrum* stained with Giemsa after acid hydrolysis for 14 min. at 60°. Note the dense single resting nucleus in each cell.

Fig. 2. Germinating conidia stained with Feulgen after acid hydrolysis for 9 min. at 60°. Note the fusion tube and the migration of an elongate nucleus.

The Growth of *Pseudomonas phaseolicola* and Related Plant Pathogens *in vivo*

By G. L. ERCOLANI*

Istituto di Patologia Vegetale, Universita degli Studi, Bologna, Italy

AND J. E. CROSSE

East Malling Research Station, Maidstone, Kent

SUMMARY

In the leaves of their natural (homologous) hosts, the bean and cherry respectively, *Pseudomonas phaseolicola* and *P. morsprunorum* increased logarithmically for at least 4 days after inoculation. Transition into the stationary phase was gradual and accompanied by water congestion of the infected tissues, followed by typical field symptoms. Increases in the inoculum dose had relatively little or no effect on the generation time but growth ceased earlier at the higher doses. In the reciprocal (heterologous) combinations, logarithmic growth was abruptly terminated after 2–3 days, due apparently to a specific defensive reaction in the host. This coincided at the higher inoculum doses with the appearance of dry necrotic symptoms in the leaves. No macroscopic symptoms were observed with the lower doses, and with the lowest dose in bean there was an acceleration of leaf maturation in the presence of heterologous organisms. Generation times were lower in heterologous combinations but increased markedly with the inoculum dose. The growth of the pear strain of *P. syringae* in bean and cherry leaves showed typical heterologous characteristics. The final yields of bacteria per unit inoculum were invariably higher in homologous combinations, but they decreased with increasing dose, whereas heterologous yields increased. The differences in net growth response were therefore greatest at the lowest doses. This suggested that host specificity in the field was associated with factors controlling growth of the organisms *in vivo* from small initial inocula.

INTRODUCTION

The 'halo blight' organism *Pseudomonas phaseolicola* is virtually restricted in the field to species of the genus *Phaseolus*, whereas the related bacterial canker organism *P. morsprunorum* is found only on plants of the genus *Prunus*. In common with many other phytopathogenic bacteria, however, both organisms when inoculated under suitable conditions will grow and induce symptoms in plants having little botanical affinity with their natural hosts (Erikson, 1945; Dye, 1958; Logan, 1960; Klement & Lovrekovich, 1961, 1962; Klement, 1963; Klement, Farkas & Lovrekovich, 1964). The following investigation compared the growth of *P. phaseolicola* and *P. morsprunorum* in natural hosts of these organisms and it was undertaken to

* Present address: Consiglio Nazionale delle Ricerche, Centro Sperimentale per la Sterilizzazione Industriale degli Alimenti, Parma, Italy.

provide preliminary information on the factors determining host specificity in the field. A strain of *P. syringae* from pear was also included in the investigation. This species was originally described from lilac but has since been identified in one form or another from thirty-nine different genera of host plants, including *Phaseolus* and *Prunus*. Pear strains of this organism are of a distinctive phage type apparently specific to this host in the field (Crosse & Garrett, 1963).

METHODS

Test plants. These were seedlings of the dwarf bean *Phaseolus vulgaris*, varieties 'Enfant de Mont Calme' and 'Canadian Wonder', and 2-year-old potted sweet cherry trees, *Prunus avium* variety 'Bigarreau Moreau'. The beans were inoculated in the primary leaves before these were fully expanded and the cherries on the first fully expanded leaves on the extension shoots as soon as sufficient of these were available to ensure adequate replication.

Bacterial isolates. The isolate *Pseudomonas phaseolicola* 1L3 was obtained from lesions on dwarf bean plants affected with halo blight in Italy in 1962. *Pseudomonas morsprunorum* was represented by a cherry strain, c22, originally isolated from leaf surfaces in Kent in 1960, and *P. syringae* by a pear strain, s14, isolated from diseased blossoms in Sussex in 1959. Stock cultures of these organisms were maintained on nutrient agar+2% (v/v) glycerol slopes (NAG) in half-ounce screw-capped bottles at approximately 4°.

Preparation of inoculum. A 24 hr growth on a NAG slope was quickly suspended in 5 ml. of sterile distilled water and immediately decanted into a sterile test tube. From this suspension a series of inocula at 10^7, 10^6, 10^5 and 10^3 bacteria/ml. was prepared, the concentration being adjusted turbidimetrically by reference to calibration curves relating extinction to numbers of viable organisms (colony counts).

Inoculation techniques. Suspensions were infiltrated through the stomata on the lower surface of the leaves by applying them in the form of fine spray delivered from a paint gun supplied with compressed air (25 lb./in.²), and held at about 6 cm. from the leaf surface. Infiltrated areas became dark green due to water congestion of the intercellular spaces, but this disappeared after 2–3 hr. leaving no visible signs of damage to the tissues. Subsequent symptom patterns invariably showed the bacteria to have been fairly uniformly distributed in the leaves, except for narrow bands of tissues running along the main veins which were rarely penetrated. On each host each treatment, i.e. bacterium/concentration combination, was applied to a separate group of plants, the number of plants in each group being sufficient to provide 20 leaves of comparable age and size for inoculation. This degree of replication and the systematic sampling procedure described below were found to be essential for obtaining growth curves of acceptable accuracy and reproducibility. After inoculation the test plants were maintained in an air-conditioned greenhouse at 20–25°.

Estimation of bacterial populations in the leaves. Four hours after inoculation the leaves were thoroughly rinsed in sterile distilled water to remove as many as possible of the bacteria adhering to the surfaces. The first sample of leaf tissue was then taken for estimation of bacterial numbers and the sampling repeated daily for 5 days, and in some instances on the seventh and tenth days after inoculation. On each sampling occasion three 5 mm. discs of tissue were cut from each of the 20 leaves under each

treatment, from sites between the main veins defined in advance on a systematic pattern. The sixty discs/treatment thus obtained were bulked, washed in two changes of sterile distilled water and homogenized in 12 ml. of sterile distilled water (SDW) for 4 min. in an MSE microhomogenizer running at 12,000 rev./min. With cherry leaves a phosphate buffer was added before homogenizing. The homogenates were allowed to stand for 1 hr with intermittent shaking.

A series of dilutions was then prepared from the supernatant fluids and 1 ml. samples of these spun in Astell roll tubes with 4·5 ml. of molten (48°) nutrient agar containing 5 % (w/v) sucrose and 1/124,000 crystal violet (British Drug Houses). In this medium *Pseudomonas phaseolicola*, *P. morsprunorum* and *P. syringae* all form characteristic radially-striated levan-type colonies (Crosse, 1959) which are readily distinguishable from colonies of contaminant organisms, although in practice the latter were rarely encountered.

Colony counts were made after 3 days at 25° and from these the numbers of bacteria/cm.² leaf tissue were calculated.

RESULTS

Three separate comparisons were made with each organism in bean and two in cherry. For simplicity, general growth trends in leaves are illustrated by the results of a single experiment in each host (Fig. 2). For all other purposes the results of replicate experiments were combined. This was justified by the high degree of reproducibility obtained. The initial inoculum dose (n 4) in each treatment was taken as the number of bacteria/cm.² leaf recovered at 4 hr. Mean generation times (m.g.t.) were calculated from values interpolated from the growth curves near the beginning and the end of the logarithmic phase. Final populations/cm.² leaf in the stationary phase (n.s.p.) were estimated by extrapolating parallel to the time axis to time 0. To obtain a measure of the period of active population increase in the leaves, the growth period, we calculated the time (t) that would have been required to reach the stationary phase in a state of continuous logarithmic growth.

It was assumed in this work that the numbers of bacteria recovered from leaves were always a constant proportion of the numbers actually present. This was confirmed by plotting log. n 4 against the log. inoculum concentration. The two were linearly related (Fig. 1), showing that for a tenfold increase in the numbers of bacteria infiltrated in the leaves, there was a tenfold increase in the numbers recovered.

General growth characteristics in vivo

Population trends. The relationship between numbers of bacteria/cm.² leaf and time is shown in Fig. 2. In no bacterium/host combination was there evidence of a lag phase beyond 4 hr except with *Pseudomonas morsprunorum* in cherry, where it was extended to between 4 and 24 hr. In all cases the populations increased logarithmically and with the homologous combinations, i.e. *P. phaseolicola* in bean and *P. morsprunorum* in cherry, entered into a stable stationary phase after about 5 days. With the heterologous combinations the transition was more abrupt and occurred earlier, usually between 2 and 3 days. The only significant departure from this pattern occurred with *P. syringae* at the highest inoculum dose in cherry. Here the maximum populations were attained at a very early stage, but these then declined

to a stable level below the stationary phase of the next lowest inoculum concentration.
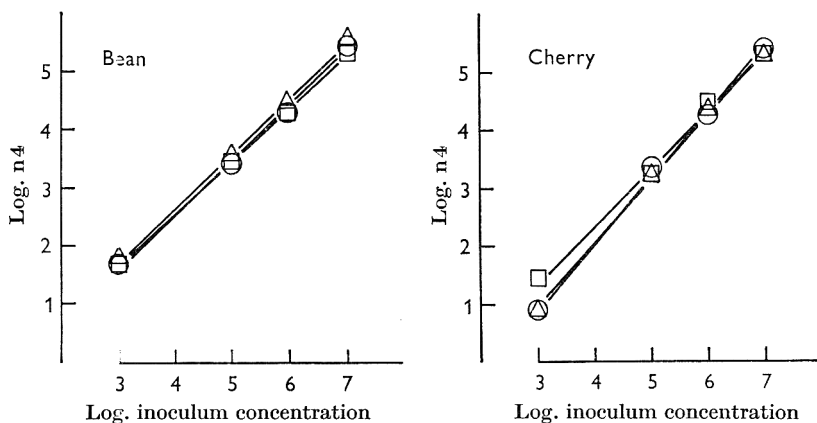
Fig. 1. Relationship between inoculum concentration and initial inoculum dose (n4) in bean and cherry leaves. *Pseudomonas phaseolicola* ($\bigcirc$), *P. morsprunorum* ($\square$) and *P. syringae* ($\triangle$).

Rate of growth. The mean generation times in the logarithmic phase varied between 5 and 6 hr for homologous bacteria and between 5 and 13 hr for the heterologous forms, according to the initial inoculum dose (Table 1). These values compare with generation times for these organisms of 1–2 hr under optimal conditions *in vitro*. The rate of growth (generations/hr) of the homologous bacteria was generally higher than that of the heterologous bacteria, but it increased less rapidly with the inoculum dose (Fig. 3). The differences were therefore less marked with the higher doses. In one of the homologous combinations, *Pseudomonas morsprunorum* in cherry, the rate of growth was more or less independent of the inoculum size. The rate of growth of the heterologous organisms increased approximately linearly with log. n4 except for *P. syringae* in cherry, where there was an abnormal increase in the growth rate at the highest inoculum dose to a value above that of the corresponding

Table 1. *Mean generation times of Pseudomonas phaseolicola, P. morsprunorum and P. syringae in bean and cherry leaves*

Pathogen	Inoculum concentration	Mean generation time (hr):	
		in bean	in cherry
P. phaseolicola	10^3	5·48*	13·32
	10^5	5·32*	9·07
	10^6	5·05*	7·93
	10^7	4·78*	6·93
P. morsprunorum	10^3	13·28	6·23*
	10^5	9·60	6·18*
	10^6	7·97	6·43*
	10^7	6·83	6·13*
P. syringae	10^3	13·13	9·08
	10^5	7·95	7·43
	10^6	6·32	6·78
	10^7	5·25	5·28

* Homologous combinations.
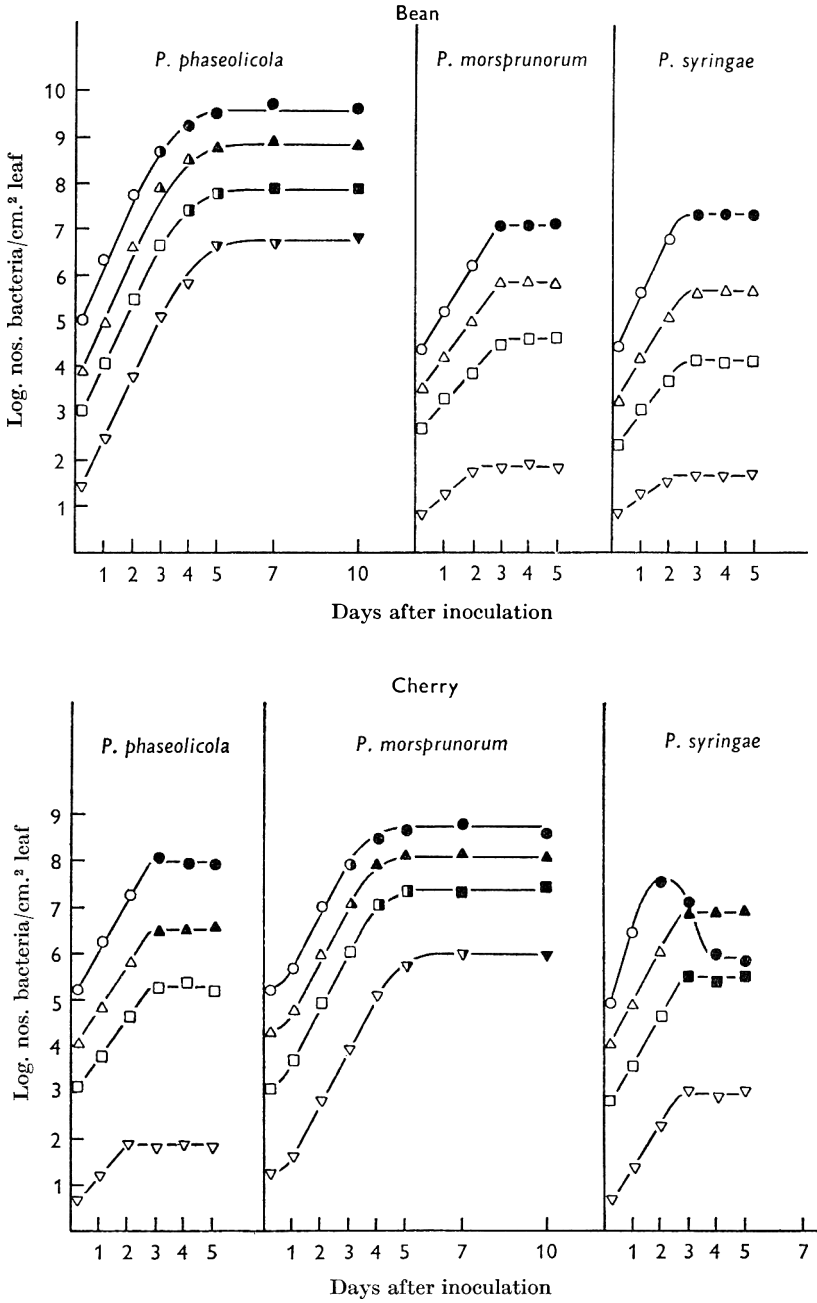
Fig. 2. Log. nos. bacteria in bean and cherry leaves at various intervals after inoculation. Inoculum concentrations (bacteria/ml.) :10^3 ($\triangledown$), 10^5 ($\square$), 10^6 ($\triangle$), 10^7 ($\bigcirc$). Half-closed symbols indicate first appearance of water soaking in tissues and closed symbols first appearance of necrotic symptoms.

homologous organism. This was observed in duplicate experiments and in both cases it was associated with early inhibition of logarithmic growth followed by a decline in populations.

Growth periods. These varied from about 100 to 70 hr for homologous bacteria, and from about 69 to 43 hr for heterologous bacteria, according to the size of the initial inoculum dose. In homologous combinations the growth period, t, decreased fairly regularly as the dose was raised, but there was no consistent relationship between t and dose in the heterologous combinations (Fig. 4). Thus in three heterologous combinations the shortest growth period occurred at the lowest dose; there was a marked extension of the growth period at the next dose, but further increase in the dose had either no effect (*Pseudomonas phaseolicola* in cherry) or decreased the growth period only fractionally (*P. syringae* and *P. morsprunorum* in bean). In the
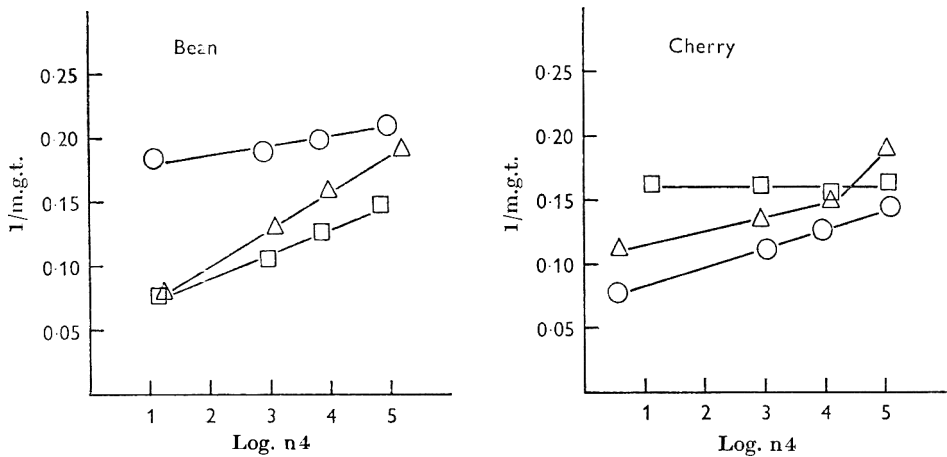
Fig. 3. Mean no. generations/hr (1/m.g.t.) in the logarithmic phase of *Pseudomonas phaseolicola* (○), *P. morsprunorum* (□) and *P. syringae* (△) after inoculation at various inoculum doses (n4) into bean and cherry leaves.
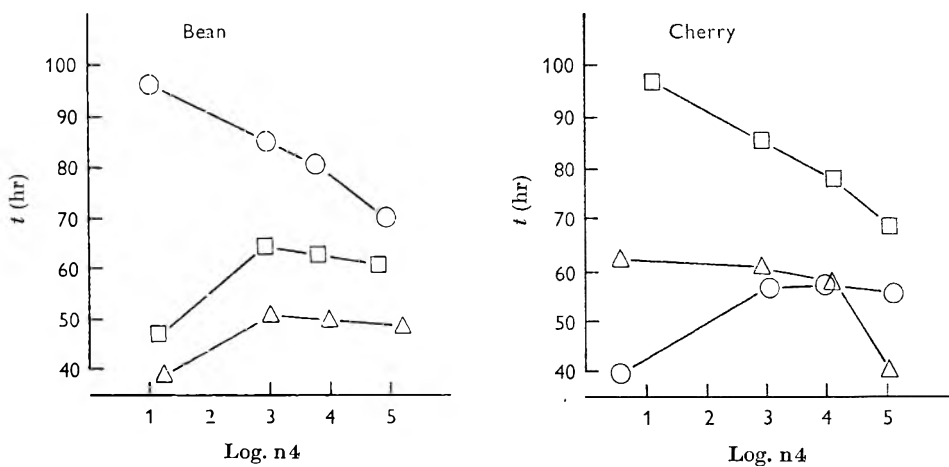
Fig. 4. Effect of inoculum dose (n4) on the growth period (*t*). *Pseudomonas phaseolicola* (○), *P. morsprunorum* (□) and *P. syringae* (△).

fourth combination (*P. syringae* in cherry) the situation was reversed, the longest growth period occurring at the lowest dose; increase in the dose had little effect except at the highest dose when there was a significant shortening of the growth period.

The abrupt termination of heterologous growth has been noted by other investigators and attributed to a specific inhibitory reaction in the host (Allington & Chamberlain, 1949; Klement & Lovrekovich, 1962; Klement *et al.* 1964). Its occurrence in bean and cherry leaves was completely unrelated to the concentration of bacteria in the tissues at the time. For example, the final populations (n.s.p.) at the end of the logarithmic phase of *P. phaseolicola* in cherry at the lowest dose were $4\cdot3 \times 10/cm.^2$ leaf tissue, and at the higher doses $1\cdot2 \times 10^5$, $2\cdot2 \times 10^6$ and $5\cdot7 \times 10^7/cm.^2$, respectively. The corresponding values of t were 44, 61, 61 and 60 hr, respectively.
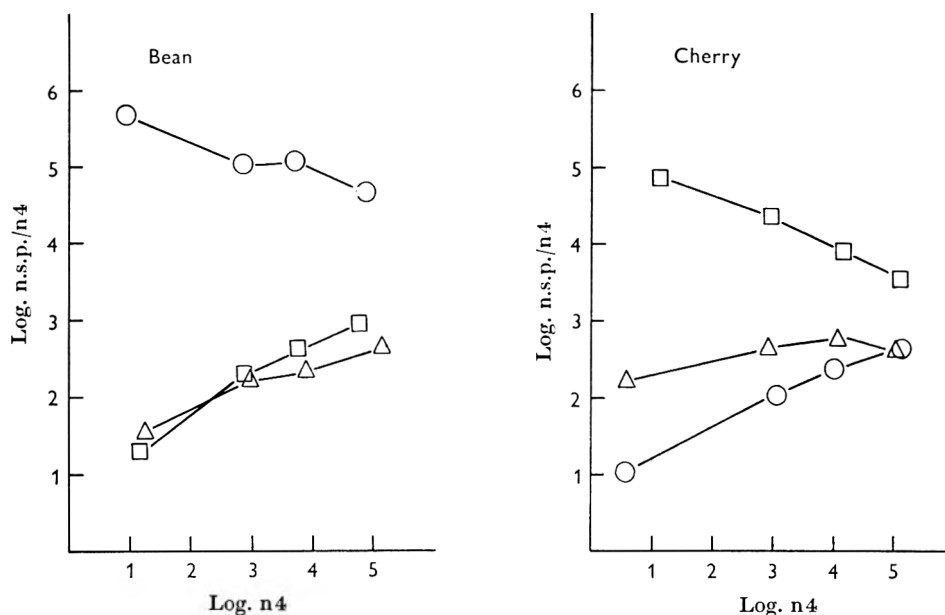
Fig. 5. Effect of inoculum dose (n4) on the final yield of bacteria per unit inoculum (n.s.p./n4). *Pseudomonas phaseolicola* ($\bigcirc$), *P. morsprunorum* ($\square$) and *P. syringae* ($\triangle$).

Growth yields. The yield per unit inoculum (n.s.p./n4) of the bacteria in homologous and heterologous combination is shown in Fig. 5. This summarizes the net growth response of the organisms at different doses. The most striking feature was that the yield of heterologous bacteria increased with dose while that of the homologous bacteria decreased. The difference in relative efficiency with which the leaf substrate was utilized for growth was thus most marked at the lower doses. The magnitude of the difference is illustrated by the yields per unit inoculum of *Pseudomonas phaseolicola* and *P. morsprunorum* in bean. These were $4\cdot4 \times 10^5$ and $1\cdot9 \times 10$, respectively, at the lowest dose; $4\cdot7 \times 10^4$ and $9\cdot3 \times 10^2$ at the highest dose. The homologous/heterologous productivity ratios, i.e. relative yields, for these particular combinations were therefore approximately 232,000:1 at the lowest dose, but only 51:1 at the highest dose.

28-2

Since the growth rates of homologous bacteria were not markedly affected by the dose size, the yields at different doses were primarily determined by the length of the growth period. The yield of heterologous bacteria, on the other hand was primarily determined by the growth rate, except occasionally at extreme doses where there was an effect of dose on the growth period.

Symptom development

Mature symptoms consisted of brown and desiccated necrotic areas in the intervcinal regions of the leaf. With heterologous bacteria they were produced after 2–3 days and approximately coincided with the abrupt transition from logarithmic to stationary growth (closed symbols in Fig. 2). The incubation period was longer with homologous bacteria and the mature symptoms were always preceded by the
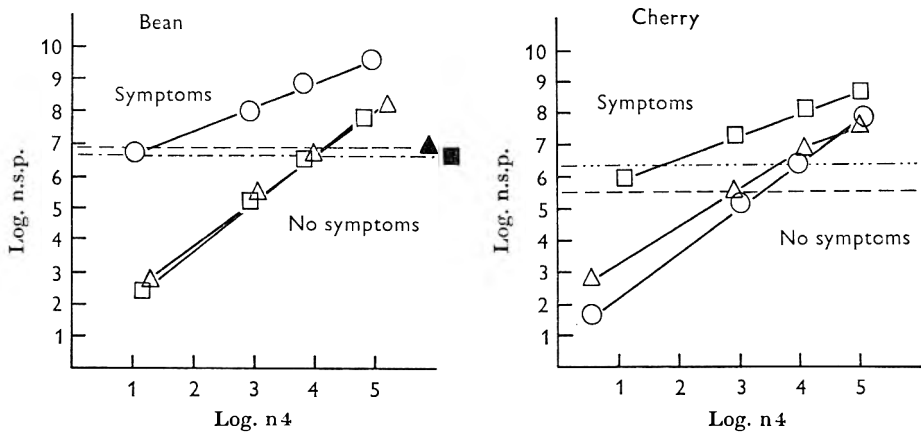
Fig. 6. Relationship between symptom production, final bacterial populations/cm.^{2} leaf in the stationary phase (n.s.p.), and the inoculum dose (n4). *Pseudomonas phaseolicola* ($\bigcirc$), *P. morsprunorum* ($\square$) and *P. syringae* ($\triangle$). Closed symbols indicate minimum final populations for symptom production in heterologous bacterium/host combinations.

development of olive-green water-soaked areas in the affected tissues during the transition period of growth (half-closed symbols in Fig. 2). The total time for production of mature symptoms of homologous bacteria varied from 4 days at the highest inoculum dose to between 7 and 10 days at the lowest dose. The latter resulted in discrete circular spots reminiscent of field infection. At higher doses the spots became confluent and large continuous areas of tissues were destroyed. Confluent necrosis was observed only with heterologous bacteria at the highest inoculum dose; there were no symptoms at the lower doses. These differences were related to the concentration of bacteria in the stationary phase (n.s.p.; Fig. 6). The broken horizontal lines in Fig. 6 indicate the lowest values of n.s.p. observed in the experiments for symptom production by an heterologous organism. The minimum value was always exceeded by homologous bacteria but by the heterologous organisms only at the higher inoculum doses.

Effect of in vivo growth on the expansion of primary leaves

The introduction of pathogenic bacteria into the intercellular spaces of immature leaves usually resulted in a visible check to further leaf expansion as compared with the uninoculated controls. Exceptions to this occurred with the lowest concentrations of the heterologous organisms, *Pseudomonas morsprunorum* and *P. syringae* in bean, where there was evidence of stimulatory effect. This is illustrated in Fig. 7, which shows the percentage increase or decrease in leaf area (maximum length × maximum width) as compared with controls when the primary leaves of bean plants were inoculated with different concentrations of the heterologous organism. The results for duplicate plants are shown separately and the values are the means for the two primary leaves on each plant.
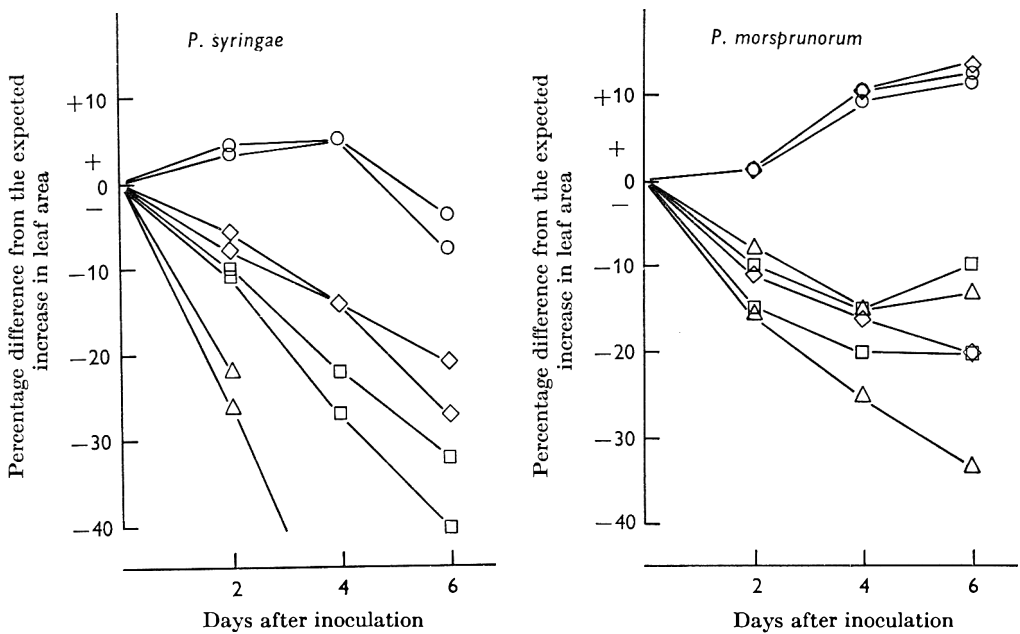
Fig. 7. Effect of heterologous organisms *Pseudomonas syringae* and *P. morsprunorum* at various inoculum concentrations on the expansion of primary leaves of bean. Inoculum concentrations (bacteria/ml.): 10^3 (○), 10^5 (◇), 10^6 (□), 10^7 (△).

The most striking effect was with the lowest concentration of *Pseudomonas morsprunorum* where the relative area of the leaves increased progressively during the first 6 days after inoculation by > 10 % over that of the uninoculated controls. A similar effect was observed in one duplicate plant at 10^5 bacteria/ml., but in the other replicate there was a depression of leaf growth. With *P. syringae*, the stimulatory effect at the lowest inoculum concentration persisted for 4 days, after which the effect was reversed and the final leaf area was slightly less than the controls. At all other inoculum concentrations intercellular growth of *P. syringae* and *P. morsprunorum* checked or retarded leaf expansion to a degree related to the inoculum concentrations.

DISCUSSION

The abrupt termination of the logarithmic growth phase of heterologous pseudo-monas organisms in bean and cherry leaves agreed with results of other workers and must be similarly attributed to specific bacteriostatic responses induced in the host (Allington & Chamberlain, 1949; Klement & Lovrekovich, 1961, 1962). Whether it was the same as the hypersensitivity reaction described by Klement *et al.* (1964) in tobacco leaves is less certain, since it was not accompanied at the lowest inoculum doses by visible necrosis of the leaf tissues. Necrosis may have occurred at the cell level and was not detected at the surface of the leaves. It is difficult, however, to reconcile damage to the tissues with the increased rate of maturation which followed the introduction of heterologous bacteria into bean leaves at the lowest inoculum concentration.

The lack of relationship between the time of appearance of the inhibitory response and the initial and final bacterial populations in the leaves also agrees with results in other heterologous combinations (Klement & Lovrekovich, 1961, 1962; Klement *et al.* 1964) and it suggests that induction of the response may be largely independent of the numbers of bacteria in the tissues. This is consistent with recent evidence which indicates that induction occurs soon after inoculation and is followed by a latent period before the inhibitory effect appears (Klement & Goodman, personal communication). Except in a few instances at extreme doses the inhibitory effect in bean and cherry leaves appeared at a relatively constant interval after inocula-tion. This could be explained by postulating that the length of the latent period is primarily determined by host reactions which proceed after induction indepen-dently of bacterial growth. However, it is clear that additional factors were operat-ing at the extreme doses. At the lowest inoculum dose in bean one such factor may have been the more rapid maturation of the leaves. The physiological changes associated with this process are well known to increase the resistance of tissues to pathogenic bacteria ('mature-leaf resistance'). Possibly this accelerates or short-circuits host reactions during the latent period.

The lower growth rate of heterologous bacteria in the logarithmic phase compared with homologous forms has not been previously reported. In bean and cherry leaves it was apparent soon after inoculation and hence, if due to plant inhibitors, these were either induced very rapidly, or were present before infection. It is worth noting in this connexion that Klement & Lovrekovich (1962) postulated the presence of pre-infection inhibitors to explain the failure of the saprophytic pseudomonas species to grow in plant tissues. Alternatively the slower growth of the heterologous forms may have been due to their inability to mobilise adequate leaf nutrients. Bacterial growth is predominantly intercellular but depends ultimately on the extent to which mesophyll cells of the plant can be rendered permeable to intra-cellular nutrients. The water soaking of tissues which invariably preceded the pro-duction of necrotic symptoms by homologous pseudomonads indicated drastic changes in cell permeability in the presence of these organisms. There was no evidence of a comparable effect in the presence of heterologous pseudomonads.

The growth of homologous organisms approximated more closely to growth *in vitro* and there were no signs in the growth curves of any inhibitory host reaction comparable to that induced by the heterologous forms. They also differed from the heterologous forms by producing progressively lower yields per unit inoculum with

each successive dose increase. This was primarily due to the shortening of the logarithmic phase and not to changes in the generation time. Thus the growth of homologous bacteria was not markedly affected until they had reached relatively high concentrations in the tissues, when it is probable that they begin to compete for a dwindling supply of leaf nutrients. The shorter logarithmic periods at the higher doses, therefore, may simply reflect the attainment of nutritionally competitive population levels at an earlier stage than at the lower doses.

At all inoculum doses homologous organisms grew more efficiently than heterologous forms, but the difference was greatest at the lowest inoculum dose, and there is little doubt that it would have been even greater if the inoculum dose had been reduced even further. There are strong implications here that host specificity in the field may be related to factors controlling *in vivo* growth of these organisms from very small inocula. In the field the stone fruit pathogen, *Pseudomonas morsprunorum*, occurs as an epiphyte on the leaves of its host (Crosse, 1959) and is mobilized in rain to give inoculum which Shanmuganathan (1962) has estimated to vary between 10^2 and 10^5 bacteria/ml. under optimal disease conditions. This is the same order of concentration as the lowest experimental inocula. In the experiments, however, inoculum was infiltrated into leaves until all the intercellular spaces were filled and the leaves visibly water-congested. Penetration never occurs on this scale in the field, so that the actual inoculum dose in natural infection can only be a fraction of those used here experimentally. At these concentrations, it is clear that heterologous organisms would not induce disease symptoms. Indeed it is doubtful whether they would grow in leaves at all. Further information is required on the minimum effective dose for *in vivo* growth. In the meantime extrapolation from the present results suggests that wheras this may be very low for homologous organisms, considerably higher numbers of bacteria would be required to initiate *in vivo* growth of heterologous organisms.

REFERENCES

ALLINGTON, W. B. & CHAMBERLAIN, D. W. (1949). Trends in the population of pathogenic bacteria within the leaf tissues of susceptible and immune plant species. *Phytopathology* **39**, 656.

CROSSE, J. E. (1959). Bacterial canker of stone-fruits. IV. Investigation of a method for measuring the inoculum potential of cherry trees. *Ann. appl. Biol.* **47**, 306.

CROSSE, J. E. & GARRETT, C. M. E. (1963). Studies on the bacteriophagy of *Pseudomonas mors-prunorum*, *P. syringae* and related organisms. *J. appl. Bact.* **26**, 159.

DYE, D. W. (1958). Host specificity in Xanthomonas. *Nature, Lond.* **182**, 1813.

ERIKSON, D. (1945). Certain aspects of resistance of plum trees to bacterial canker. II. On the nature of bacterial invasion of *Prunus* sp. by *Pseudomonas mors-prunorum* Wormald. *Ann. appl. Biol.* **32**, 112.

KLEMENT, Z. (1963). Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature, Lond.* **199**, 299.

KLEMENT, Z. & LOVREKOVICH, L. (1961). Defence reactions induced by phytopathogenic bacteria in bean pods. *Phytopath. Z.* **41**, 217.

KLEMENT, Z. & LOVREKOVICH, L. (1962). Studies on host–parasite relations in bean pods infected with bacteria. *Phytopath. Z.* **45**, 81.

KLEMENT, Z., FARKAS, G. L. & LOVREKOVICH, L. (1964). Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. *Phytopathology* **54**, 474.

LOGAN, C. (1960). Host specificity of two Xanthomonas species. *Nature, Lond.* **188**, 479.

SHANMUGANATHAN, N. (1962), *Studies in bacterial canker of plum and cherry* (*Pseudomonas mors-prunorum* Wormald). Ph.D. Thesis, University of London.

Cholesterol Oxidation by Soil Actinomycetes

By R. L. BROWN* and G. E. PETERSON

Department of Biology, University of Houston, Houston, Texas, U.S.A.

SUMMARY

Cholesterol enrichment cultures from soil samples yielded various *Nocardia* and *Streptomyces* spp. capable of metabolizing cholesterol aerobically as a sole carbon source. One of these isolates, designated *Streptomyces* 14PH8, formed haloes (cholesterol-free zones surrounding colonies) on cholesterol mineral salts agar. This organism was selected for further study as it was able to utilize 80–100 % of 0·1 % (w/v) cholesterol in a mineral salts medium in 6 days. Oxidation of the sterol was initiated by a cholesterol dehydrogenase, giving 4-cholestene-3-one. The latter compound was then hydroxylated to form 4-cholestene-4-ol-3-one. Isotopic tracer studies revealed all of carbon-4 and most of carbon-26 of cholesterol were converted to $^{14}CO_2$. However, some of carbon-26 was converted to cell material.

Streptomyces 14PH8 gave several variants, one of which (*Streptomyces* 14PH8 no. 2 var. A) could be reclassified as a Nocardia, using the same method of Gordon & Smith (1955).

INTRODUCTION

In 1913 Sohngen first reported growth of soil micro-organisms in a cholesterol medium. Haag (1927) then demonstrated cholesterol utilization by a soil mycobacterium. Both these investigators used growth as the sole criterion of cholesterol utilization. It remained for Tak (1942) to show chemical evidence that a soil mycobacterium was actually metabolizing the sterol. Turfitt (1943, 1944, 1948) investigated microbial metabolism of cholesterol using *Nocardia erythropolis*, demonstrating metabolic changes in a cholesterol containing medium, as well as several intermediary metabolites of cholesterol degradation. Several other investigators have shown results similar to those of Turfitt (Kramli & Horvath, 1947; Schatz, Savard & Pintner, 1949; Arnaudi, 1951, 1954; Stadtman, Cherkes & Anfinson, 1954).

By cholesterol enrichment techniques Lewis (1962) isolated several streptomycetes which could utilize cholesterol as a sole carbon source. These micro-organisms were more efficient at degrading cholesterol than any described in the papers above, degrading some 80 mg. cholesterol in only 6 days.

Using certain metabolic inhibitors, Whitmarsh (1964) demonstrated several intermediate metabolites not previously known.

This paper is meant to be an extension of the work on the catabolism of cholesterol by micro-organisms. Evidence is presented for the metabolism of cholesterol as a sole carbon source by several streptomycetes.

* Present address: Department of Microbiology, The University of Texas Medical Branch, Galveston, Texas.

METHODS

Media. The composition of the mineral salts culture solution, as described by Turfitt (1948), was as follows (g./l.); KNO_3, 2·0; K_2HPO_4, 0·25; $MgSO_4.7H_2O$, 0·25; NaCl, 0·005; $FeSO_4.7H_2O$, 0·0001; agar (where used), 15; made up in tap water, 1 l.; adjusted to pH 7·0.

Cholesterol mineral salts agar was prepared by using the dispersion technique of Peterson, Lewis & Davis (1962). Cholesterol was always sterilized separately from the aqueous medium, since it has been shown that the sterol undergoes auto-oxidation when treated in aqueous media (Peterson *et al.* 1962).

Organisms. Organisms were obtained from soil samples as follows: samples of soil (5 g.) were washed with 10 ml. sterile tap water, centrifuged, and the precipitate was poured into 50 ml. cholesterol mineral salts medium. This was incubated at 27° on a rotary shaker at 180 rev./min. After incubation for 6 days, 0·1 ml. samples were removed and streaked on several cholesterol mineral salts agar plates, which were incubated at 27° for 6 days. Isolated colonies were then subcultured to purify and placed in stock cultures on tubes of the same type agar. These organisms were compared to stock cultures kept at this institution as to their cholesterol utilization and classification characteristics.

Growth. Inocula for metabolic experiments were prepared on plates containing mineral salts agar $\pm 0.2\%$ (w/v) cholesterol. These were incubated for 6 days at 27°. The growth was then suspended in 24 ml. sterile tap water; 2 ml. of this suspension was used to inoculate each metabolism flask.

Metabolism flasks were made by aseptically transferring 50 ml. sterile mineral salts solution to 250 ml. Erlenmeyer flasks containing 50 mg. sterile cholesterol. The metabolism flasks were incubated on a rotary shaker at 27° and 180 rev./min. Duplicate flasks were sampled at 0, 1, 2, 4 and 6 days of incubation. At the time of removal, the contents of each flask were extracted with 25 ml. chloroform. The chloroform and aqueous layers were separated and stored at 4° until analysed.

Cholesterol and 4-cholestene-3-one determinations. Cholesterol was determined by the Sperry & Webb (1950) modification of the Lieberman–Burchard reaction.

4-Cholestene-3-one was determined by the method of Stadtman *et al.* (1954) for detecting phenylhydrazone derivatives.

Classification of organisms. Classification of organisms under study was accomplished by the method of Gordon & Smith (1955).

Isotopic tracer studies. Twenty-five μc of 4-^{14}C-cholesterol were added to a Waring blendor containing 300 ml. of sterile mineral salts solution and blended for 2 min. This was repeated in another blendor flask but with 26-^{14}C-cholesterol. The content of each blendor were poured into a separate, stoppered, 50 ml. metabolism flask and inoculated with aerial mycelia washed from two 6-day Kollé plates containing 0·2% (w/v) cholesterol mineral salts agar. Metabolism was allowed to proceed at 27°. A constant supply of sterile air was bubbled into the flasks at 5 lb./in.[2]. Output CO_2, produced by biological activity, was trapped in an attached small flask which contained 150 ml. 0·1 N-NaOH. At 24 hr intervals, 10 ml. samples were removed from each of the metabolism flasks and extracted with 5 ml. chloroform. Two ml. of each of the chloroform extracts were placed in separate deep planchettes (2 ml.) and counted, after being dried, for 5 min. on a Nuclear Chicago Model 181 A

counter. At the same time, 5 ml. of the NaOH solution was removed, precipitated with 5 ml. 0·1 M-BaCl$_2$, and the precipitate collected on a sintered steel filter containing a uniform layer of 6 mg. Celite; these were dried and counted as just described. The organisms were filtered, washed with chloroform, dried and counted in planchettes as described for the carbonate collection. The cell-free aqueous layers were treated and counted as described in the procedure for the chloroform extracts.

Identification of 4-cholestene-4-ol-3-one. The presence of 4-cholestene-4-ol-3-one was ascertained by chromatography, absorption spectra, and melting-point determinations as described below. Ultraviolet absorption spectra were determined by evaporating 25 ml. of the chloroform extract from pooled metabolism flasks, dissolving the residue in ethanol, and obtaining the spectrum on a Beckman Model DB spectrophotometer. Chromatograms were made by using Silica gel H (250 μ layers) developed with solvents 9 + 1 (w/v) benzene ethanol (Randerath, 1963) and a 4 + 1 chloroform ethyl acetate (Bennet & Heftman, 1962). The functional hydroxyl group was detected by using a 5 % (w/v) phosphomolybdic acid (in absolute ethanol) spray reagent; the ketone group was detected by using a phenylhydrazine spray reagent. Melting points were determined by adding 1 % (w/v) digitonin (in ethanol) and allowing the mixture to stand for 18 hr. The digitonides were collected by centrifugation and added to 25 ml. pyridine and extracted with ether. This extract was streaked in a solid line across the bottom of a Silica gel G plate (250 μ layer) and developed to the 10 cm. mark with the 4 + 1 (by vol.) chloroform ethyl acetate solvent. The area around R_F 0·97 was scraped from the plate, taken up in chloroform, and filtered free from Silica gel. After drying, the melting point of the resulting crystals was taken on a Fisher melting-point apparatus.

The synthesis of 4-cholestene-4-ol-3-one was by the method of Fieser (1954).

Cholesterol dehydrogenase. Studies of the enzyme cholesterol dehydrogenase, first shown in these organisms by Davis, Brown, Lewis & Peterson (1964), were made by filtering 250 ml. of 6-day metabolism liquor through sintered glass filters. Twenty-five ml. of this cell-free liquor was then inoculated into each of six 250 ml. Erlenmeyer flasks, each containing 10 mg. sterile cholesterol. These flasks were incubated at 27° and 180 rev./min. on a rotary shaker. Two flasks were removed at 0, two at 3 days, and two at 6 days. The contents of each flask were extracted with 10 ml. chloroform. After separation, the layers were stored at 4° until all flasks had been removed, at which time all flasks were assayed for cholesterol and 4-cholestene-3-one. Before extraction 0·01 ml. from each flask was inoculated into 10 ml. nutrient broth, allowed to incubate at 27° for 3 days, and observed for growth. The appearance of turbidity was taken to indicate that the enzyme preparations contained viable organisms. These, where found, were discarded.

RESULTS

The metabolism of cholesterol by soil actinomycetes is shown in Figs. 1 and 2. A comparison is shown between streptomycetes and nocardias.

Classification of organisms. The results of the classification studies are shown in Table 1. Comparisons are shown to known stock cultures kept at this institution. *Streptomyces* K1PH7 and K2PH7 were found in soils near Kerrville, Texas, and the others were taken from the soil near Houston, Texas. *Streptomyces* 14PH8 no. 2A

was a variant obtained by serial enrichment culture of the parent isolate 14PH8 on cholesterol mineral salts medium. Serial transfers of *Streptomyces* 14PH8 at weekly intervals to fresh cholesterol mineral salts medium were made for a period of several
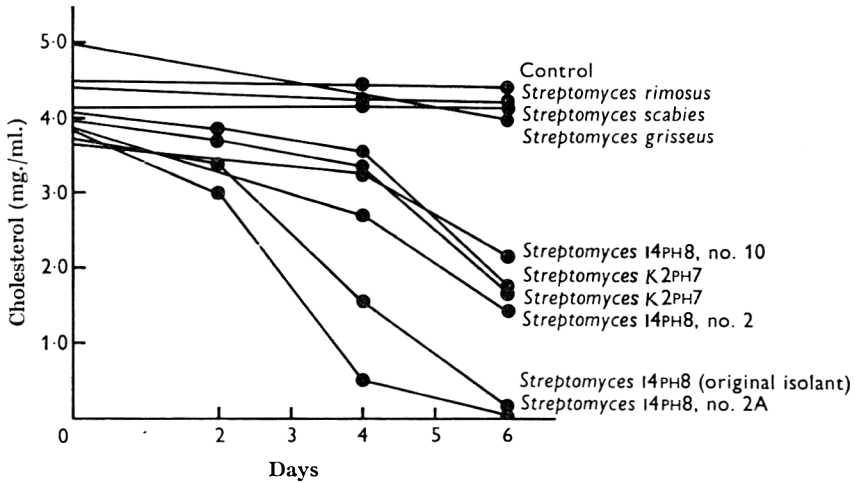
Fig. 1. Metabolism of cholesterol by various streptomyces species.
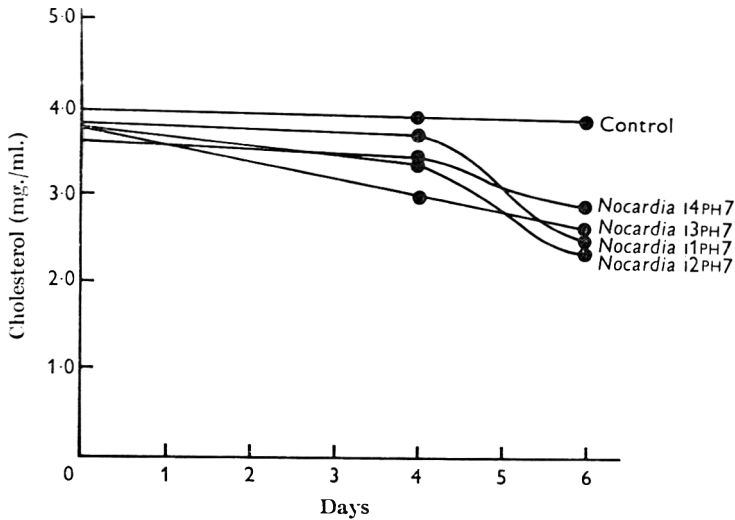
Fig. 2. Metabolism of cholesterol by various nocardia species.

months. At this time the organism used more cholesterol than ever before, but its biochemical and morphological characteristics had changed. It now grew only very sparsely on cholesterol mineral salts agar, although growth in liquid cholesterol medium and output of cholesterol dehydrogenase was greatly enhanced. Further, according to the same procedure of Gordon & Smith (1955), it now more nearly resembles a nocardia.

The metabolic changes wrought by the action of *Streptomyces* 14PH8 on chol-
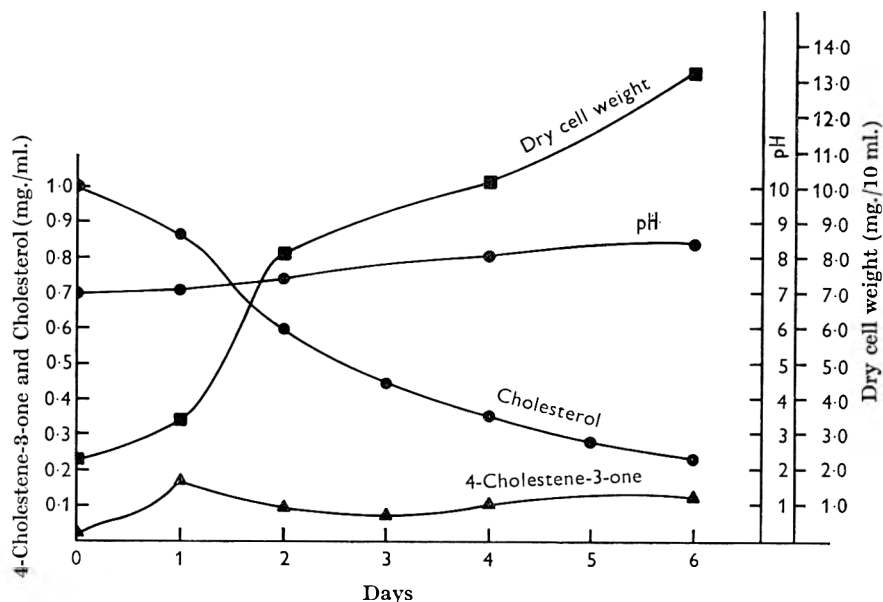
Fig. 3. Biochemical changes occurring in cholesterol mineral salts liquor as a result of growth of *Streptomyces* 14PH8.

Table 1. *Characteristics of soil isolates according to the procedure of Gordon & Smith (1955)*

	Lect-ose	Malt-ose	Xyl-ose	Mann-ose	Decom-position of tyro-sine	Hydro-lysis of casein	Aerial mycelia	Spores	Acid fast-ness	Halo forma-tion
Streptomyces 14PH8	A*	A	A	A	+	+	+	+	+	+
Streptomyces 14PH 8 no. 2	A	A	A	A	+	−	−	Sli.†	−	+
Streptomyces 14PH 8 no. 10	A	A	−	A	+	−	Decr.‡	+	−	−
Streptomyces 14PH 8 no. 2 var. A	−	−	−	A	+	−	−	−	−	−
Nocardia 11PH 7	−	−	−	−	−	−	+	+	−	−
Nocardia 12PH 7	−	−	A	−	−	−	+	+	−	−
Nocardia 13PH 7	−	−	−	A	+	−	Rud.§	−	Sl.‖	−
Nocardia 14PH 7	−	−	−	−	−	−	+	+	−	−
Streptomyces K1PH 7	−	A	−	A	+	−	+	+	−	−
Streptomyces K2PH 7	A	A	−	A	+	−	+	+	Sl.	−
Streptomyces 13PH 4	A	A	A	A	+	−	+	+	+	−

* A = acid.   † Sli. = slime former.   ‡ Decr. = decreased.   ‖ Sl. = slight.
§ Rud. = rudimentary.
Halo formation was not included in the method of Gordon & Smith (1955).
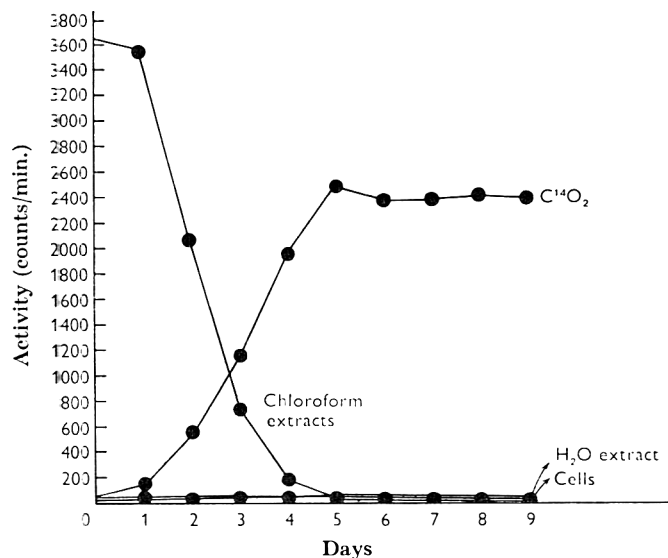
Fig. 4. Distribution of activity of 26-^{14}C-cholesterol as a result of growth of *Streptomyces* 14pH8 no. 2.
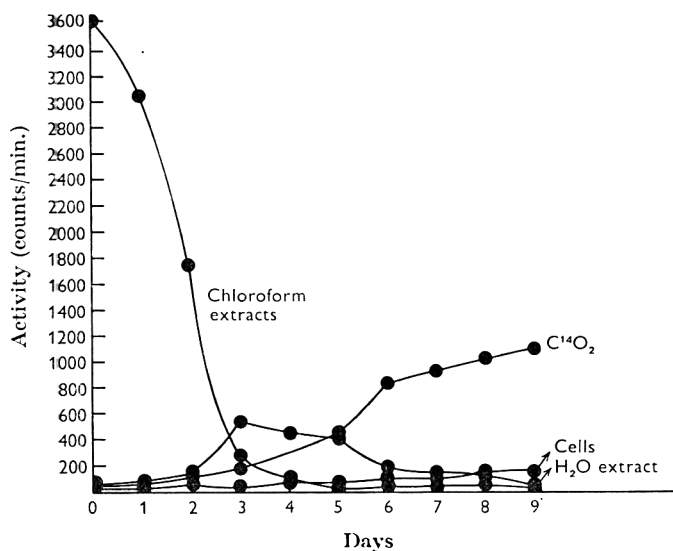
Fig. 5. Distribution of activity of 4-^{14}C-cholesterol as a result of growth of *Streptomyces* 14pH8 no. 2.

Table 2. *Oxidation of cholesterol by cell-free extracts of Streptomyces 14PH8 no. 2 var. A*

Days of incubation	Residual cholesterol (mg./ml.)	4-cholestene-3-one produced (mg./ml.)
0	0·441	0
3	0·345	0·100
6	0·295	0·231

esterol are shown in Fig. 3. The decrease in cholesterol content of the medium was accompanied by a corresponding increase in the amount of 4-cholestene-3-one, which was further metabolized to cell material.

Cholesterol dehydrogenase. The activity of the enzyme cholesterol dehydrogenase is depicted in Table 2. The amount of 4-cholestene-3-one produced was, within experimental limits, equal to the amount of cholesterol degraded.
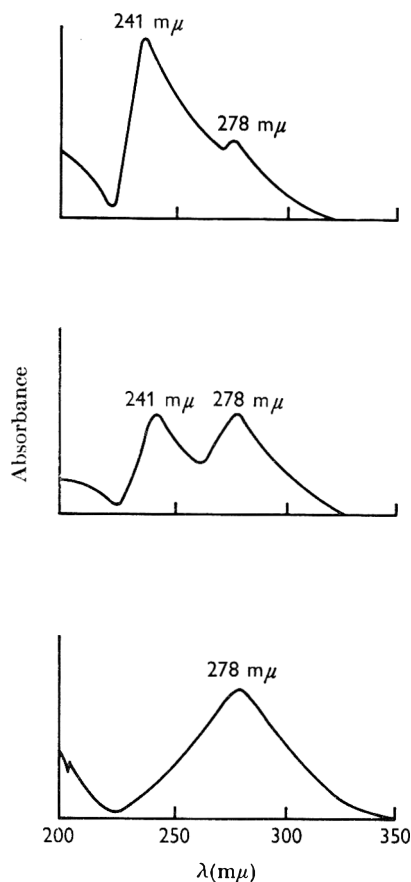
Fig. 6. Ultraviolet absorption spectra. Top: chloroform extract of cholesterol mineral salts liquor after 6-day incubation with *Streptomyces* 14PH8 no. 2. Centre: a 1/10 dilution of the same extract, with added 4-cholestene-4-ol-3-one. Bottom: spectrum of pure 4-cholestene-4-ol-3-one.

Isotopic tracer studies. Figures 4 and 5 show the radioactivity of both 4-^{14}C-cholesterol and 26-^{14}C-cholesterol to be converted mostly to CO_2. In addition, 26-^{14}C-cholesterol was also metabolized to some water soluble component as well as to cell components.

Identification of 4-cholestene-3-one and 4-cholestene-4-ol-3-one. Ultraviolet absorption spectra are shown in Fig. 6, which shows the spectra of 4-cholestene-4-ol-3-one, a suspected intermediate, and a mixture of the two, indicating identical absorption maxima. The suspected intermediate had a melting point of 150·5°, which is compatible with authentic 4-cholestene-4-ol-3-one.

Table 3 shows thin layer chromatographic comparisons of 6-day metabolism extracts with 4-cholestene-3-one and 4-cholestene-4-ol-3-one. The extracts show spots compatible with the two standards used. One intermediate, 4-cholestene-3-one, contains a ketone group and absorbs u.v. radiation maximally at 241 mμ. The second intermediate, suspected of being 4-cholestene-4-ol-3-one, contains both a ketone and a hydroxyl group and has the same R_f values as does the known compound. The ketone group was shown by the use of a phenylhydrazine spray reagent, and the hydroxyl function showed bright blue when sprayed with a 5 % (w/v) phosphomolybdic acid solution.

Table 3. *Thin layer chromatograms of 6-day fermentation extracts of Streptomyces 14PH8 grown on cholesterol-mineral salts broth*

R_F							
Extracts		Cholesterol		4-cholestene-3-one		4-cholestene-4-ol-3-one	
1*	2†	1*	2†	1*	2†	1*	2†
0·76	0·93				0·95	0·75	0·95
0·52	0·67		0·65	0·50			
0·25		0·25					

* Developed with a 9:1 benzene–ethanol solvent system.
† Developed with a 4:1 chloroform–ethyl acetate solvent system.

DISCUSSION

From the data presented, it can be seen that carbon-4 and carbon-26 were both decarboxylated, with carbon-4 going entirely to CO_2 and carbon-26 being at least partially taken into cell components. Further tracer studies, which would allow us to determine the fate of other carbons, were hampered by the unavailability of other ^{14}C-labelled compounds.

The pathway by which isolate 14PH8 breaks down the cholesterol molecule is apparently begun by oxidation of the molecule to 4-cholestene-3-one. This in turn hydroxylated at 4-carbon position to give 4-cholestene-4-ol-3-one, which is further metabolized by decarboxylation. No other intermediates have as yet been found. In view of this 4-carbon decarboxylation, one might postulate a link between similar species of micro-organisms, such as the similarity between the organism used at this institution and the *Nocardia erythropolis* of Turfitt (1948). In addition, there is also some similarity to the Mycobacteria of Stadtman *et al.* (1954). This organism produced an extracellular cholesterol dehydrogenase which degraded cholesterol to 4-cholestene-3-one. This was then degraded via loss of carbon-4 and carbon-26; all of these reactions are in common with those found at this institution. Among other compounds, Turfitt found his Nocardia to produce Windaus's keto acid, which could result from decarboxylation of 4-cholestene-4-ol-3-one. A likely mechanism could be a CD2-A decarboxylation of Diels's acid, or, less likely, dihydro Diels's acid.

Cholesterol dehydrogenase from *Streptomyces* 14PH8 no. 2 converted some 37 % of the cholesterol sample to cholestenone in cell-free extracts with a corresponding (near-quantitative) increase in 4-cholestene-3-one.

Problems continuously asserted themselves in the inherent instability of *Streptomyces* 14PH8. The parent strain gave two variants, *Streptompces* 14PH no. 2 and 14PH8 no. 10, which differed from the original isolate, as shown in Table 1. The no. 2 strain retained its halo-forming ability but formed a slime on a more complete medium (such as Bennet's agar or tomato paste oatmeal agar). This was in contrast to the parent strain, which formed gray spores on the complete medium. Loss of the ability to form haloes was accompanied by a decrease in the amount of cholesterol the organism could metabolize. The exception to this is seen in *Streptomyces* 14PH8 no. 2A, which is by far the most active microorganism in our stocks on cholesterol-containing mineral salts medium. This variant not only did not form haloes, but grew only very sparsely on cholesterol mineral salts agar. Just why this is seen is unknown, but apparently the long-term enrichment culture used to attain this variant resulted in the selection of a particular strain (or heterocaryon) which grew better in liquid media than on solid media. This variant now resembles a *Nocardia* sp. more closely than the parent *Streptomyces* sp. This may indicate a closer link between the two groups than previously suspected.

REFERENCES

ARNAUDI, C. (1951). Oxydations du microbiennes des steroids. *Experientia* 5, 120.

ARNAUDI, C. (1951). Research on the microbiological degradation of steroids. *Appl. Microbiol* 2, 274.

BENNET, R. D. & HEFTMAN, E. (1962). Thin layer chromatography of corticosteroids. *J. Chromatog.* 9, 348.

DAVIS, J. R., BROWN, R. L., LEWIS, H. L. & PETERSON, G. E. (1964). Cholesterol utilization by *Streptomyces* spp. *Bact. Proc.* p. 100.

FIESER, L. G. & STEVENSON, R. (1954). Cholesterol and companions. Oxidation of 5-cholestene-3-one with lead tetraacetate. *J. Am. chem. Soc.* 76, 1731.

GORDON, R. E. & SMITH, M. M. (1955). A proposed group of characters for the separation of *Streptomyces* and *Nocardia. J. Bact.* 69, 147.

HAAG, F. (1927). Die saphrophytischen Mycobacterien. *Zentbl. Bakt. ParasitKde* Abt. II 71, 1.

KRAMLI, A. & HORVATH, J. (1947). Microbiological oxidation of cholesterol with *Azotobacter. Nature, Lond.* 160, 639.

LEWIS, H. L. (1962). Evidence for cholesterol metabolism by soil streptomycetes. *M.S. Thesis. University of Houston, Texas.*

PETERSON, G. E., LEWIS, H. L. & DAVIS, J. R. (1962). Preparation of uniform dispersions of cholesterol and other water insoluble carbon sources in agar media. *J. lipid. Res.* 3 275.

PETERSON, G. E., MANDY, W. J., FUTCH, H. & LUCKEY, D. (1962). Cholesterol deposition in *Penicillium* spp. *Can. J. Microbiol.* 8, 193.

RANDERATH, K. (1963). *Thin Layer Chromatography.* New York: Academic Press.

SCHATZ, A., SAVARD, K. & PINTNER, I. (1949). The ability of soil microorganisms to decompose cholesterol. *J. Bact.* 58, 117.

SÖHNGEN, N. L. (1913). Benzin, Petroleum, Paraffinöl and Paraffin als Kohlenstoff-und Energiequelle für Mikroben. *Zentbl. Bakt. ParasitKde* (Abt. II), 37, 595.

SPERRY, W. M. & WEBB, M. (1950). A revision of the Schoenheimer–Sperry method for cholesterol determination. *J. biol. Chem.* 187, 97.

STADTMAN, T. C., CHERKES, A. & ANFINSON, C. B. (1954). Studies on the microbiological degradation of cholesterol. *J. biol. Chem.* 206, 511.

Tak, J. D. (1942). On bacteria decomposing cholesterol. *Antonie van Leeuwenhoek* **8**, 32.

Turfitt, G. E. (1943). The microbiological degradation of steroids. I. The sterol content of soils. *Biochem. J.* **37**, 115.

Turfitt, G. E. (1944). The microbiological degradation of steroids. II. Oxidation of cholesterol by *Proactinomyces* spp. *Biochem. J.* **38**, 492.

Turfitt, G. E. (1948). Microbiological agencies in the degradation of steroids. II. Steroid utilization by the microflora of soils. *J. Bact.* **54**, 557.

Whitmarsh, J. M. (1964). Intermediates of microbiological metabolism of cholesterol. *Biochem. J.* **90**, 23 pp.

Comparative Studies on the Agglutination of Fowl Red Blood Cells by the Haemagglutinins of Vaccinia and Coxsackie A7 Viruses

By J. D. WILLIAMSON*

*Viral Epidemiology Unit of the University of Glasgow,
Ruchill Hospital, Glasgow, Scotland*

SUMMARY

Red blood cells from a group of fowls were sensitive to the haemagglutinins of vaccinia and Coxsackie A7 viruses but the red cells from certain fowls exhibited differences in their relative sensitivities. Differences between the reaction of these haemagglutinins with susceptible red cells of the same sensitivity were shown by treatment with chemical and physical agents. There were differences in the effect of pH value on the haemagglutination titres and, unlike Coxsackie A7 haemagglutinin, the adsorption of vaccinia haemagglutinin to susceptible red cells was inhibited by divalent cations. Red cell receptors for both haemagglutinins were insensitive to RDE (receptor destroying enzyme) but were inactivated by treatment with potassium periodate, papain or α-chymotrypsin. There were quantitative differences in the degrees or rates of receptor destruction by these reagents. Haemagglutination by vaccinia and Coxsackie A7 haemagglutinins was inhibited only by homologous antiserum. These qualitative and quantitative differences indicate separate red cell receptors for the two haemagglutinins.

INTRODUCTION

Red blood cells from certain fowls are highly susceptible to agglutination by the haemagglutinin specifically associated with vaccinia-infected tissue. Other fowls and most other animals possess red cells which are either agglutinated to a much decreased titre or are completely insensitive (Clark & Nagler, 1943; Burnet & Boake, 1946). Fowl red cells which are agglutinable by the vaccinia haemagglutinin show a parallel sensitivity to agglutination by normal tissue lipids (Burnet & Stone, 1946). Physicochemical studies have shown that vaccinia haemagglutinin is particulate and distinct from the infective particle (Chu, 1948; Neff, Ackermann & Preston, 1965). The activity of this haemagglutinin is associated with lipid and it is probably a lipoprotein (Stone, 1946a; Chu, 1948).

The restriction of the range of sensitive red cells to 'lipid-sensitive' fowl red cells appeared to be a phenomenon peculiar to vaccinia and other poxvirus haemagglutinins (Burnet & Boake, 1946; McCarthy & Helbert, 1960). However, Grist (1960) described a haemagglutinin specifically associated with tissue infected with an enterovirus, the Coxsackie A7 virus, which will also agglutinate only vaccinia-agglutinable

fowl red cells. This Coxsackie A 7 haemagglutinin was shown to be particulate and separable from the infective particle. Unlike vaccinia haemagglutinin, the activity of the Coxsackie A 7 haemagglutinin appears not to be associated with lipid but with protein (Williamson & Grist, 1965).

Although both haemagglutinins will agglutinate only 'lipid-sensitive' fowl red cells, differences in the chemical nature of the reactive sites of vaccinia and Coxsackie A 7 haemagglutinins suggested that they might attach to susceptible red cells by different mechanisms. This hypothesis has been investigated by comparative studies of the reactions of these haemagglutinins with susceptible fowl red cells.

METHODS

Viruses. The viruses used were the Evans strain of vaccinia virus and the 1034 strain of Coxsackie A 7 (Grist, 1960); sometimes the Lee strain of influenza type B virus was also used.

Preparation of haemagglutinins. Extracts of suckling mouse tissue infected with Coxsackie A 7 virus were prepared as described previously (Williamson & Grist, 1965) and used without further treatment as Coxsackie A 7 haemagglutinin.

Stock suspensions of vaccinia virus passaged in chick embryos were used to inoculate the chorioallantoic membranes of 12-day chick embryos to give confluent lesions. Membranes were removed 48 or 72 hr later and extracted with physiological saline by disruption of the infected membranes in a mechanical homogenizer. The extracts were clarified by low-speed centrifugation and the supernatant fluid used as vaccinia haemagglutinin.

The Lee strain of influenza type B virus was passaged in the allantoic cavities of 10-day chick embryos. The influenza virus haemagglutinin was obtained by harvesting infected allantoic fluid 48 hr after inoculation.

Haemagglutination and haemagglutination inhibition tests. The preparation of vaccinia-agglutinable fowl red blood cells (r.b.c.) and the performance of these tests in Perspex depression trays were described by Williamson & Grist (1965). Haemagglutination inhibition tests were used routinely to test the specificity of vaccinia haemagglutinin preparations. The haemagglutinin specifically associated with vaccinia-infected tissue is inhibited by vaccinia antiserum but not by normal serum (Stone, 1946 a).

Preparation of antisera. Cockerels were immunized by four intramuscular injections at weekly intervals of 1·0 ml. of an extract of suckling mouse tissue infected with Coxsackie A 7 virus, emulsified with an equal volume of incomplete Freund's adjuvant. The fowls were bled 7 days after the final injection.

Vaccinia antisera were prepared similarly in fowls by injection with adjuvant of an extract of vaccinia-infected chick embryo chorioallantoic membrane.

Treatment of red cells. It was established in the early stages of these investigations that vaccinia-agglutinable fowl r.b.c. were rapidly rendered auto-agglutinable by even limited exposure to RDE, potassium periodate or proteolytic enzymes. This prohibited the quantitative assessment of the effect of treatment by direct use of these cells in haemagglutination tests. In order to overcome this problem, the following indirect procedure was adopted.

Washed vaccinia-agglutinable fowl r.b.c. were resuspended to 1 % (v/v) in dupli-

cate serial dilutions of 5·0 ml. amounts of 0·0025 M-potassium periodate in physiological saline. Similar duplicate 1 % (v/v) r.b.c. suspensions were made in 0·01 % trypsin, 0·1 % α-chymotrypsin, 1 % papain and *Vibrio cholerae* filtrate (Burroughs Wellcome Ltd.). The proteolytic enzymes were dissolved in phosphate buffer saline, 0·14 M-sodium chloride +0·01 M-phosphate buffer (pH 7·2). Papain was activated by addition of 3 % (w/v) cysteine hydrochloride. *Vibrio cholerae* filtrate was reconstituted as directed by the suppliers. Treatment with KIO_4 was at 18° for 1 hr; with proteolytic enzymes at 37° for up to 6 hr; with *V. cholerae* filtrate at 37° overnight. Controls included in each series consisted of r.b.c. at a similar concentration incubated at the appropriate temperature in the absence of the particular reagent. After treatment, the r.b.c. were deposited by centrifugation at 500 *g* for 5 min. and washed three times with 0·85% NaCl. Periodate-treated r.b.c. were washed with 0·85 % NaCl containing 1 % (w/v) glucose.

One of the duplicate sets of treated r.b.c. was then resuspended in 1·0 ml. of a solution containing four haemagglutinating units of Coxsackie A7 haemagglutinin. The other set was resuspended in 1·0 ml. of a solution containing vaccinia haemagglutinin at a similar concentration. Mixtures, together with controls, were kept at 18° for 2 hr with resuspension of the r.b.c. at frequent intervals. After exposure to the haemagglutinins, the r.b.c. were deposited by low-speed centrifugation and the titre of haemagglutinating activity remaining in the supernatant fluids determined.

RESULTS

Range of susceptible fowl red cells

The sensitivity of washed r.b.c. from 172 Rhode Island Red fowls to vaccinia and Coxsackie A7 haemagglutinins was determined. Suspensions of r.b.c. were adjusted to 0·5 % (v/v) by using a haematocrit. Serial twofold dilutions of each haemagglutinin were made from stock preparations containing 16 haemagglutinating units when titrated with the same susceptible r.b.c. All dilutions were made in bulk before dispensing on to Perspex plates in order to make each dilution series strictly comparable. Results were read 2 hr after the addition of 0·5 % (v/v) fowl r.b.c. suspensions and again after standing at 4° overnight.

Of the 172 samples of fowl r.b.c. tested, 60 % gave identical titres with both haemagglutinins, 17 % gave a twofold lower titre and 17 % gave a twofold or slightly higher titre with Coxsackie A7 haemagglutinin than with vaccinia haemagglutinin. There was an eightfold difference between the maximal and minimal titres obtained with both haemagglutinins. The remaining 6 % of the fowl r.b.c. tested were not agglutinated even by higher concentrations of either haemagglutinin. Insensitive r.b.c. were also obtained from 12 chick embryos which were tested.

The fowl r.b.c. used in the experiments reported here were equally sensitive to both vaccinia and Coxsackie A7 haemagglutinins.

Effect of physical agents on haemagglutination

Temperature. Haemagglutination titres obtained with vaccinia and Coxsackie A7 haemagglutinins were similarly affected by the temperature at which the tests were made. Parallel tests showed a twofold increase at 37° and a twofold decrease at 4° as compared with titres obtained at room temperature (15–18°). The patterns of

agglutinated r.b.c. at each temperature differed, however, in that vaccinia haemagglutinin gave a uniformly distributed shield pattern, whereas Coxsackie A7 haemagglutinin gave a more densely packed pattern of coarsely agglutinated r.b.c.

Electrolytes. Vaccinia and Coxsackie A7 haemagglutinins were dialysed overnight against isotonic solutions of glucose, NaCl, $MgCl_2$, $CaCl_2$ or Na_2SO_4. Haemagglutination titres were then determined with the use of isotonic solutions for the preparation of 0·5 % (v/v) fowl r.b.c. suspensions. With both vaccinia and Coxsackie A7 haemagglutinins, haemagglutination was not detectable in the absence of electrolytes. Coxsackie A7 haemagglutinin gave identical haemagglutination titres in each of the isotonic salt solutions. Identical haemagglutination titres were obtained with vaccinia haemagglutinin in isotonic solutions of NaCl and Na_2SO_4. Haemagglutination with the vaccinia haemagglutinin, however, was decreased 16-fold in isotonic $MgCl_2$ and was completely inhibited in isotonic $CaCl_2$. Unlike Coxsackie A7 haemagglutinin, agglutination of susceptible fowl r.b.c. by vaccinia haemagglutinin was inhibited by the divalent cations tested.

In other experiments, vaccinia haemagglutinin of a known haemagglutination titre in 0·85 % NaCl solution was dialysed overnight against isotonic $CaCl_2$. Susceptible fowl r.b.c. equilibrated to isotonic $CaCl_2$ were added to the dialysed preparation and the mixture kept at room temperature for 2 hr with resuspension of the r.b.c. at frequent intervals. The r.b.c. were then deposited by low-speed centrifugation and the supernatant fluid dialysed against isotonic NaCl. Haemagglutination titres determined after dialysis indicated complete recovery of vaccinia haemagglutinin in the supernatant. In control experiments with isotonic NaCl as the suspending medium, haemagglutinin was not detectable in the supernatant fluid following exposure to susceptible r.b.c. Ca^{2+} ions therefore prevented the adsorption of vaccinia haemagglutinin to susceptible fowl r.b.c. Further experiments established that Ca^{2+} ions similarly inhibited agglutination of susceptible r.b.c. by a 0·4 % (w/v) suspension of lecithin prepared as described by Stone (1946b).

Effect of pH values. Haemagglutination titres were determined at room temperatures in 0·85 % NaCl buffered with citrate buffers (pH 5·0–6·0), phosphate buffers (pH 7·0–8·0) and glycine buffers (pH 9·0–10·0). Preliminary experiments had shown that both haemagglutinins and fowl r.b.c. were stable over these ranges. There were marked differences in the effect of the pH value of the diluent on the titres obtained with vaccinia and Coxsackie A7 haemagglutinins (Fig. 1). With Coxsackie A7 haemagglutinin, maximal titres were obtained between pH 5·0 and 8·0 but there was a marked decrease in titre as the diluent became progressively more alkaline. Conversely, titres were minimal in acid diluents with the vaccinia haemagglutinin, maximal titres being obtained in the alkaline range. Titres obtained with vaccinia haemagglutinin were closely paralleled by those obtained with the lecithin preparation under the same conditions.

Effect of treatment of fowl red blood cells

Vibrio cholerae filtrate. A receptor destroying enzyme (RDE) present in *V. cholerae filtrate* destroys the red cell receptors for influenza virus (Burnet & Stone, 1947). Treatment of lipid-sensitive fowl r.b.c. with an RDE preparation under the conditions described destroyed their ability to absorb the type B influenza virus strain

Lee, but was without effect on the receptors for the vaccinia and Coxsackie A 7 haemagglutinins.

Potassium periodate. Although r.b.c. receptors for vaccinia and Coxsackie A 7 haemagglutinins were destroyed by the highest concentration of periodate used (0·0025 M), there were quantitative differences in the degree of receptor destruction at lower concentrations (Fig. 2). Thus, susceptible fowl r.b.c. treated with 0·001 M-potassium periodate completely absorbed the Coxsackie A 7 haemagglutinin, whereas only 50 % of the vaccinia haemagglutinin was absorbed. Untreated r.b.c. completely absorbed both haemagglutinins under the same conditions. These results
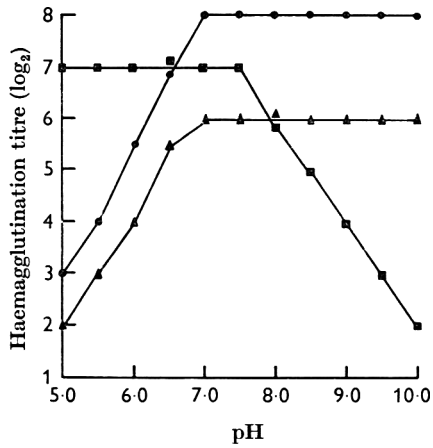
Fig. 1   Fig. 2

Fig. 1. Effect of pH value on the agglutination of lipid-sensitive fowl red blood cells by vaccinia haemagglutinin, Coxsackie A 7 haemagglutinin and a 0·4 % (w/v) lecithin suspension. ●—●, Vaccinia haemagglutinin; ■—■, Coxsackie A 7 haemagglutinin; ▲—▲, lecithin suspension.
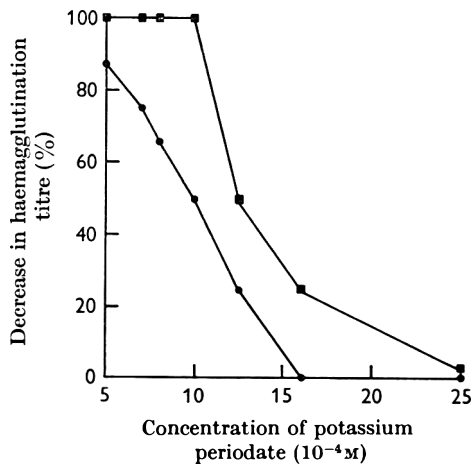
Fig. 2. Absorption of vaccinia and Coxsackie A 7 haemagglutinins by potassium periodate-treated fowl red blood cells. ●—●, Vaccinia haemagglutinin; ■—■, Coxsackie A 7 haemagglutinin.

suggest that the r.b.c. receptors for vaccinia haemagglutinin are more sensitive to the action of periodate than those for Coxsackie A 7 haemagglutinin.

Proteolytic enzymes. Papain and α-chymotrypsin destroyed receptors for both vaccinia and Coxsackie A 7 haemagglutinins but quantitative differences were again established. Under the conditions described, treatment with 1 % papain for 2 hr completely destroyed the capacity of susceptible r.b.c. to absorb vaccinia haemagglutinin. Identically treated r.b.c., however, absorbed 75 % of the Coxsackie A 7 haemagglutinin to which they were exposed. Similarly, r.b.c. receptors for vaccinia haemagglutinin were completely destroyed by 0·1 % α-chymotrypsin after 2 hr, but such r.b.c. retained the capacity to absorb 50 % of the Coxsackie A 7 haemagglutinin. Extended treatment with papain or with α-chymotrypsin rendered r.b.c. completely insensitive to Coxsackie A 7 haemagglutinin.

Treatment of r.b.c. with 0·01 % trypsin up to 6 hr did not diminish their capacity

to absorb both haemagglutinins. Modification of the conditions of treatment by increasing the incubation period or the concentration of enzyme rendered the r.b.c. unsuitable for absorption experiments.

Exclusion experiments

Attempts were made to saturate susceptible r.b.c. with one of the haemagglutinins and then to determine whether such r.b.c. were capable of absorbing the second haemagglutinin. Saturation of intact r.b.c. with Coxsackie A7 haemagglutinin was prevented by lysis of the r.b.c. by the haemolysin present in extracts of suckling mouse tissue infected with Coxsackie A7 virus (Williamson & Grist, 1965). To overcome this difficulty experiments were made with stromata prepared by lysis of susceptible r.b.c. in distilled water. Such stromata were repeatedly resuspended in preparations of either the vaccinia or Coxsackie A7 haemagglutinin. After thorough washing in 0·85 % NaCl solution at 4°, the stromata were resuspended in dilutions of the homologous haemagglutinin containing 4 haemagglutinating units. Failure to decrease the haemagglutination titre indicated complete saturation. After further washing, the stromata were resuspended in similar dilutions of the heterologous haemagglutinin. Neither stromata saturated with vaccinia haemagglutinin nor stromata saturated with Coxsackie A7 haemagglutinin showed any detectable absorption of heterologous haemagglutinin.

Stromata saturated with vaccinia or Coxsackie A7 haemagglutinin were also resuspended in dilutions of the influenza virus preparation containing 4 haemagglutinating units. Although control preparations completely absorbed the influenza virus haemagglutinin, there was no detectable adsorption to stromata saturated with vaccinia or Coxsackie A7 haemagglutinin.

Table 1. *Haemagglutination inhibition tests with vaccinia and Coxsackie A7 haemagglutinins using homologous and heterologous immune sera*

	Serum titres			
	Vaccinia		Coxsackie A7	
Haemagglutinin	Normal	Immune	Normal	Immune
Vaccinia	32	1024	32	32
Coxsackie A7	< 32	< 32	< 32	512

Haemagglutination inhibition tests

Differences in the serological specificity of vaccinia and Coxsackie A7 haemagglutinins were established by haemagglutination inhibition tests (Table 1). Homologous immune sera inhibited both haemagglutinins to high titre but there was no specific inhibition of either haemagglutinin by heterologous immune sera.

DISCUSSION

Quantitative differences revealed in the relative sensitivity of certain fowl r.b.c. to the two haemagglutinins suggest that although there is a close relationship between sensitivity to vaccinia and Coxsackie A7 haemagglutinins, there are quantitative differences in the capacity of some fowl r.b.c. to absorb these haemag-

glutinins. Failure of Coxsackie A7 haemagglutinin to agglutinate chick embryo r.b.c. indicates that red cell receptors for this haemagglutinin appear only as the fowls mature as previously described for receptors for vaccinia haemagglutinin (McCarthy & Helbert, 1960).

The qualitative and quantitative differences in the effect of physical and chemical agents on the reaction of vaccinia and Coxsackie A7 haemagglutinins with susceptible fowl r.b.c. reflect differences in the physicochemical properties of both the haemagglutinins and the red cell receptors. Inhibition by divalent cations of the agglutination of r.b.c. by lipid-associated haemagglutinins has been previously reported with vaccinia haemagglutinin (Briody, 1951) and with the psittacosis haemagglutinin (Gogolak & Ross, 1955). In the present study, only vaccinia and lecithin haemagglutinins were inhibited by either Ca^{2+} or Mg^{2+} ions. Physical and chemical studies have indicated that the activity of the Coxsackie A7 haemagglutinin is not associated with lipid but with protein (Williamson & Grist, 1965).

The results of treatment of r.b.c. with RDE, periodate and proteolytic enzymes suggest that the red cell receptors are probably mucoproteins but distinct from myxovirus receptors (Andrewes, Bang & Burnet, 1955). Quantitative experiments indicate that, if the receptors are chemically similar, there are more receptors for the Coxsackie A7 haemagglutinin than for the vaccinia haemagglutinin. Conversely, if there are the same number of receptors for each haemagglutinin, it may be argued that the receptors are chemically different. Attempts to resolve the relationship between the receptors by saturation experiments gave equivocal results and suggest only that there may be a close spatial proximity between the receptors for the two haemagglutinins. However, either interpretation of the quantitative experiments points to a distinction between the receptors for vaccinia and Coxsackie A7 haemagglutinins.

Previous studies have shown that there are differences in the chemical nature of the red cell receptors for the haemagglutinins in the main groups of viruses (Klenk & Lempfrid, 1957; Buckland & Tyrell, 1963; Philipson *et al.* 1964). In addition to chemical differences (Williamson & Grist, 1965), vaccinia and Coxsackie A7 haemagglutinins have been shown to be serologically distinct. Both qualitative and quantitative differences have been established in the reaction of these haemagglutinins with susceptible fowl r.b.c. It is concluded that these results indicate that, although closely linked, there are separate red cell receptors for vaccinia and Coxsackie A7 haemagglutinins.

REFERENCES

Andrewes, C. H., Bang, F. B. & Burnet, F. M. (1955). A short description of the myxovirus group (influenza and related viruses). *Virology* 1, 176.

Briody, A. (1951). A 'unitarian' view of vaccinia haemagglutinin. *Yale J. Biol. Med.* 24, 1.

Buckland, F. E. & Tyrell, D. A. J. (1963). A comparative study of virus haemagglutinins. The stability of haemagglutinins and red cell receptors to certain chemical and physical treatments. *J. gen. Microbiol.* 32, 241.

Burnet, F. M. & Boake, W. C. (1946). The relationship between the virus of infectious ectromelia of mice and vaccinia virus. *J. Immunol.* 53, 1.

Burnet, F. M. & Stone, J. D. (1946). The haemagglutinins of vaccinia and ectromelia viruses. *Aust. J. exp. Biol. med. Sci.* 24, 1.

Burnet, F. M. & Stone, J. D. (1947). The receptor destroying enzyme of *V. cholerae. Aust. J. exp. Biol. med. Sci.* 25, 227.

Chu, C. M. (1948). Studies on vaccinia haemagglutinin: some physicochemical properties. *J. Hyg., Camb.* 46, 42.

Clark, E. & Nagler, F. P. O. (1943). Haemagglutination by viruses. The range of susceptible cells with special reference to agglutination by vaccinia virus. *Aust. J. exp. Biol. med. Sci.* 21, 103.

Gogolak, F. M. & Ross, M. R. (1955). The properties and chemical nature of psittacosis virus haemagglutinin. *Virology* 1, 176.

Grist, N. R. (1960). Isolation of Coxsackie A7 virus in Scotland. *Lancet*, i, 1054.

Klenk, E. & Lempfrid, H. (1957). Über die Natur der Zellreceptoren für das Influenza virus. *Z. Physiol. Chem.* 307, 278.

McCarthy, K. & Helbert, D. (1960). A comparison of the haemagglutinins of variola, alastrim, vaccinia, cowpox and ectromelia viruses. *J. Path. Bact.* 79, 416.

Neff, B. J., Ackermann, W. W. & Preston, R. E. (1965). Studies of vaccinia haemagglutinin obtained from vaccinia infected tissue. Density gradient centrifugation and electron microscopy. *Proc. Soc. exp. Biol. Med.* 118, 664.

Philipson, L., Bengtsson, S., Brishammar, S., Svennerholm, L. & Zetterqvist, Ö. (1964). Purification and chemical analysis of the erythrocyte receptor for haemagglutinating enteroviruses. *Virology* 22, 580.

Stone, J. D. (1946a). Inactivation of vaccinia and ectromelia virus by lecithinase. *Aust. J. exp. Biol. med. Sci.* 24, 191.

Stone, J. D. (1946b). Lipid haemagglutinins. *Aust. J. exp. Biol. med. Sci.* 24, 197.

Williamson, J. D. & Grist, N. R. (1965). Studies on the haemagglutinin present in Coxsackie A7 virus-infected suckling mouse tissue. *J. gen. Microbiol.* 41, 283.

Influence of the State of Fimbriation on Transmission of the Colicinogenic Factor *colI* between Strains of *Shigella flexneri*

By M. MULCZYK* and J. P. DUGUID

Bacteriology Department, University of St Andrews, Queen's College, Dundee

SUMMARY

The colicinogenic factor *colI* derived from *Shigella sonnei* colicine type 2 (Abbott & Shannon, 1958) was transmitted between different strains of *Shigella flexneri* in serotypes 1a, 1b, 2a, 2b, 3a, 3c, 4a, 4b, 4c, 5a, X and Y when donor and acceptor strains were grown together in mixed culture in aerobic static broth for a period of 20 hr or 48 hr at 37°. The rate of transfer was influenced by the state of fimbriation of the acceptor bacteria, but not by that of the donor bacteria. The proportion of acceptor organisms acquiring *colI* was very high, e.g. 15–40 % in 20 hr and 50–95 % in 48 hr, when the acceptor was genotypically fimbriate and in the fimbriate phase (Fim$^+$F); but it was much lower, e.g. 0–5 % in 20 hr and 1–15 % in 48 hr, when the acceptor was either genotypically non-fimbriate (Fim$^-$) or genotypically fimbriate but in the non-fimbriate phase (Fim$^+$N). Although it facilitated the transmission of *colI*, the presence of fimbriae was not essential for transmission, since transmission at a low rate was obtained in most crosses of Fim$^-$ donors with Fim$^-$ acceptors. Only 1 out of 17 strains of *S. flexneri* tested as acceptors failed to acquire *colI* in any test. Transmission did not take place within the first 2 hr of mixed culture; it occurred mainly during the period of slow growth after the end of the exponential phase (i.e. between 8 and 48 hr) when, with fimbriate organisms, a pellicle of bacteria had developed on the surface of the broth. The rate of transmission was very much decreased when pellicle formation was prevented by intermittent or continuous agitation of the culture. Experiments in which the donor bacteria were killed by adding streptomycin at 8 hr showed that an extensive 'epidemic' spread of *colI* took place within the acceptor population during the later stages of culture. When stationary-phase (1–10 days) broth cultures of donor and acceptor bacteria were mixed together and incubated without the addition of fresh broth, the rates of transfer at 20 and 48 hr were as high as in the mixed cultures grown from small inocula. Transmission also occurred with high frequency in a defined medium containing glucose, NH_4^+ and nicotinic acid.

INTRODUCTION

Many strains of bacteria in different genera of Enterobacteriaceae possess the ability to produce and liberate colicines, antibiotic substances which are active against many other strains of enterobacteria (Fredericq, 1957, 1963). This ability may be transferred, during the conjugation of bacteria, from a colicinogenic to a

* Present address: Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland.

non-colicinogenic strain (Fredericq, 1954; Ozeki, Howarth & Clowes, 1961). The colicinogenic factor that is transferred from bacterium to bacterium has the properties of a plasmid, i.e. an autonomously reproducing cytoplasmic hereditary determinant (Lederberg, 1952), and the transfer of colicinogeny is not dependent on transfer of the bacterial chromosome as in the classical recombination phenomenon. Factors influencing the frequency of transfer of colicinogenic factor I (*colI*), which determines the production of colicine I, have been investigated by Ozeki, Stocker & Smith (1962) and Stocker, Smith & Ozeki (1963) in experiments with *Salmonella typhimurium* carrying *colI* derived from *Shigella sonnei* strain P9.

The present work was done in an attempt to discover whether the transfer of *colI* factors is influenced by the presence of fimbriae in the donor and acceptor bacteria. Fimbriae are non-flagellar filamentous appendages that are present in many strains of most genera of enterobacteria and which confer adhesive properties on the bacteria; fimbriate bacilli are known to adhere to red blood cells, causing haemagglutination, and to other kinds of animal, plant and fungal cells (Duguid, Smith, Dempster & Edmunds, 1955). It seemed possible that they might have an important role in effecting the conjugation of bacteria that is necessary for the transfer of *colI*. Cultures of *Shigella flexneri* were considered to be especially suitable for use in the investigation of the influence of fimbriation on the transfer of *colI* because both genotypically fimbriate (Fim+) and genotypically non-fimbriate (Fim−) strains occur in several serotypes of the species, and because cultures of the genotypically fimbriate strains can be grown in either a fimbriate (Fim+F) or a non-fimbriate (Fim+N) phenotype, or 'phase' (Duguid & Gillies, 1957). Comparative experiments can, therefore, be made with comparable fimbriate and non-fimbriate cultures.

METHODS

Colicinogenic factors. Most of the experiments were made with *colI* factor derived from *Shigella sonnei* strain 2, the standard colicine secretor strain no. 2 of Abbot & Shannon (1958), ref. no. 10047 of the Dysentery Reference Laboratory (DRL), Colindale, London. This factor, *colI–CT2* is referred to as *colI* throughout the present paper.

For the purpose of comparison, a few experiments were made with the different *colI* factor, designated in this paper as *colI–P9*, which had been studied by Stocker *et al.* (1963). This factor was obtained from *Salmonella typhimurium* strain LT2 *cysD–36* (*colI–P9*), which had been given the factor from *S. sonnei* strain P9, and was received from Dr B. A. D. Stocker. Both the *colI* donor strains, *S. sonnei* 2 and *S. typhimurium* LT2 *cysD–36* (*colI–P9*), were streptomycin-sensitive; the former was non-flagellate and non-fimbriate (Fim−) and the latter was flagellate and fimbriate (Fim+).

Donor and acceptor strains of Shigella flexneri. These are listed, with their serotype and fimbriation genotype, in Table 1. Streptomycin-sensitive strains were used as donors of *colI*. The colicinogenic factor had been introduced into them from *Shigella sonnei* strain 2 or *Salmonella typhimurium* strain LT2 *cysD–36* (*colI–P9*). Where subsequently, in the tables and text, a strain is described as a 'donor', it is to be understood that it carries *colI* even although it is designated by its number, e.g. 1a1, without the suffix *colI*.

Streptomycin-resistant strains were used as acceptors. All these strains were resistant to 1000 μg. streptomycin/ml. They did not produce detectable colicines and they were resistant to the colicines determined by *colI* (i.e. *colI–CT*2) and *colI–P*9. The original cultures of the acceptor strains (Table 1, second column) were colicine-resistant but streptomycin-sensitive; streptomycin-resistant mutants were derived by selection in streptomycin broth and these mutants were used as the acceptors in the experiments.

Table 1. *Donor (colicinogenic) and acceptor (non-colicinogenic)*
strains of Shigella flexneri used in the experiments

Designation of strain	Parent strain*	Serotype	Fimbriation genotype
(a) Donor strains (streptomycin-sensitive)			
1a1 *colI*	NCTC 8192	1a	+
1a1 *colI–P9*	NCTC 8192	1a	+
2a1 *colI*	NCTC 8204	2a	−
2b1 *colI*	NCTC 8518	2b	+
4a1 *colI*	Edinburgh 4a1	4a	+
4c10 *colI*	DRL 127/56	4c	−
5a3 *colI*	NCTC 8523	5a	−
(b) Acceptor strains (streptomycin-resistant)			
1a1	NCTC 8192	1a	+
1b1	Wrocław 6713	1b	−
2a1	NCTC 8204	2a	−
2a19	DRL E/56/9	2a	+
2b1	NCTC 8518	2b	+
3a106	NCTC 8664	3a	+
3c2	Wrocław 6713M†	3c	−
4a1	Edinburgh 4a1	4a	+
4a2	Wrocław 4a	4a	+
4b1	DRL 181/56	4b	+
4b3	DRL E/56/6	4b	+
4c10	DRL 127/56	4c	−
5a3	NCTC 8523	5a	−
5a4	NCTC 8524	5a	+
5a12	DRL 64/56	5a	+
x105	DRL 208/63	X	+
y3	NCTC 1169	Y	+

* Parent strains were non-colicinogenic and streptomycin-sensitive. Donor strains were derived from them by infection with *colI* or *colI-P9*, and acceptors were streptomycin-resistant mutants derived from them. NCTC = National Collection of Type Cultures, Colindale, London. DRL = Dysentery Reference Laboratory, Colindale, London.

† Strain Wrocław 6713M is a serological mutant derived from the serotype 1b strain Wrocław 6713 (Mulczyk, 1960).

Indicator of colI. The indicator strain, sensitive to colicine I and used to detect it, was *Escherichia coli* strain K12–Row (Fredericq; DRL 10044), received from Dr J. D. Abbott.

Culture media. The nutrient broth used was Difco heart infusion broth. Difco heart infusion agar plates were used with or without streptomycin (1 mg./ml.). The defined medium was the glucose ammonium salts minimal medium of Ozeki *et al.* (1962) supplemented with nicotinic acid 1 μg./ml. Trypsin was not used in media.

Fimbriate and non-fimbriate cultures. Fimbriate-phase (Fim⁺F) cultures were obtained by serial culture of Fim⁺ strains at 37° in static tubes of 10 ml. broth. Non-fimbriate-phase (Fim⁺N) cultures were obtained by serial culture of Fim⁺ strains on plates of nutrient agar (Duguid & Gillies, 1957). Before use, the cultures were tested for haemagglutinating activity with a 3 % suspension of guinea-pig red cells (method of Duguid & Gillies, 1957) to confirm that they were richly fimbriate or entirely non-fimbriate. The notation used to designate the fimbriation genotype and phenotype (phase) of donor and acceptor cultures is shown in Table 2.

Table 2. *Designations used to indicate the state of fimbriation of donor and acceptor cultures*

Designation	Fimbriation status of culture
Fim⁻	Genotype non-fimbriate
Fim⁺	Genotype fimbriate
Fim⁺N	Genotype fimbriate but phenotype (or phase) non-fimbriate
Fim⁺F	Genotype fimbriate and phenotype (or phase) fimbriate

Preparation of inocula for mixed cultures. The mixed broth cultures in which transmission of *colI* was observed were inoculated with about 10^7 bacteria of the donor strain and about 10^7 bacteria of the acceptor strain. These bacteria were taken from 24 hr broth cultures of predetermined fimbriation genotype and phenotype. Phenotypically fimbriate (Fim⁺F) cultures were obtained by carrying a Fim⁺ strain through 4 or 5 serial 48 hr cultures in broth. Genotypically fimbriate but phenotypically non-fimbriate (Fim⁺N) cultures were obtained by making 4 or 5 serial 48 hr cultures on agar plates. The final 48 hr culture of the Fim⁺F or Fim⁺N organisms was subcultured in broth for 24 hr to prepare the inoculum for the experimental mixed culture. Fim⁺N organisms remained in the non-fimbriate phase during this single 24 hr period of growth in broth. Genotypically non-fimbriate (Fim⁻) organisms were inoculated into the mixed culture from a 24 hr broth culture that had been inoculated from a 48 hr broth culture.

Tests for transmission of colI in mixed cultures. Crosses were made by growing the donor and acceptor strains in mixed culture in aerobic static broth for 20 and 48 hr periods at 37°; 10 ml. broth in a test-tube stoppered with cottonwool was incubated under ordinary aerobic conditions without disturbance by movement, mixing or artificial aeration. Because sampling of the culture for examination involved mixing and disruption of any surface pellicle, separate tubes of mixed cultures were grown from similar inocula for each time of sampling (usually 20 and 48 hr) so that the culture and pellicle remained undisturbed until the sample was taken.

After the culture had been inoculated with about 10^7 donor bacteria and about 10^7 acceptor bacteria and had been incubated for the test period, it was mixed thoroughly by drawing up and down in a pipette so that any surface pellicle and any deposit were completely and uniformly dispersed. Tenfold dilutions of the culture were then prepared in sterile saline and 0·1 ml. volumes spread on plates of streptomycin nutrient agar. After incubation for 24 hr, the plates were treated with chloroform vapour for 30 min. and then overlayed with a thin layer of nutrient agar (1 % agar). They were dried and inoculated by flooding with a broth culture of the indicator strain, *Escherichia coli* K12-Row. After overnight incubation the

proportion (%) of colonies that were colicinogenic, i.e. surrounded by zones of inhibition of growth of the indicator strain, was determined. Generally this result was based on the observation of 200–700 acceptor-strain colonies.

<div align="center">RESULTS</div>

<div align="center">

Transfer of colI between genotypically fimbriate (Fim⁺)
strains of Shigella flexneri

</div>

The first experiments were made with the streptomycin-sensitive Fim⁺ strain 1a1 of *Shigella flexneri* as donor of *colI*. This strain had been rendered colicinogenic by infection with *colI* from *S. sonnei* strain 2. Like other Fim⁺ strains of *S. flexneri*, it could be obtained in a fimbriate phase (Fim⁺F) by serial culture in broth and in a non-fimbriate phase (Fim⁺N) by serial culture on agar. The acceptor strain was a streptomycin-resistant mutant derived from the original streptomycin-sensitive non-colicinogenic stock of strain 1a1; it, too, could be obtained in a fimbriate and a non-fimbriate phase. Crosses of the donor and acceptor strains were tested by growing the two strains together in mixed cultures in tubes of 10 ml. nutrient broth

Table 3. *Frequency of transfer of colI between fimbriate-phase (F) and non-fimbriate-phase (N) organisms of the Fim⁺ strains 1a1 and 2a19 of Shigella flexneri during growth in mixed culture in aerobic static broth for 20 or 48 hr at 37°*

Tubes of 10 ml. broth were inoculated with, per tube, 10⁷ bacteria of the donor (streptomycin-sensitive) culture and 10⁷ bacteria of the acceptor (streptomycin-resistant) culture, and were then incubated without disturbance for either 20 or 48 hr; separate tubes were set up for each time of incubation. After growth, the culture was thoroughly mixed and dilutions of it plated on streptomycin agar so as to yield colonies derived only from the acceptor-strain bacteria. These colonies were tested for colicinogeny.

Cross		Viable count of streptomycin-resistant (acceptor strain) bacteria ($\times 10^{-6}$/ml.) in mixed culture at		% of acceptor-strain colonies found to be colicinogenic (and no. colicinogenic/ no. tested*) at	
Donor bacteria	Acceptor bacteria	20 hr	48 hr	20 hr	48 hr
1a1 F	1a1 F	140	95	37 (302/816)	93 (516/554)
1a1 F	1a1 N	71	42	< 0·3 (0/417)	22 (52/246)
1a1 N	1a1 F	130	89	40 (301/752)	95 (492/518)
1a1 N	1a1 N	62	46	< 0·3 (0/364)	16 (42/262)
1a1 F	2a19 F	130	66	27 (103/380)	81 (150/185)
1a1 F	2a19 N	37	33	1·4 (3/216)	4·3 (4/93)
1a1 N	2a19 F	160	150	25 (111/446)	86 (372/432)
1a1 N	2a19 N	93	78	0·8 (2/258)	1·4 (3/219)

* Numbers in parentheses are: no. of acceptor-strain colonies found to be colicinogenic/no. of acceptor-strain colonies tested.

incubated at 37° under aerobic conditions without agitation, disturbance or artificial aeration. Four crosses were made with the organisms in different combinations of phases: (1) donor Fim⁺F × acceptor Fim⁺F, (2) donor Fim⁺F × acceptor Fim⁺N, (3) donor Fim⁺N × acceptor Fim⁺F, (4) donor Fim⁺N × acceptor Fim⁺N. The results are shown in Table 3.

After 20 hr incubation of the mixed culture, *colI* had been transmitted to a large

percentage (37 and 40 %) of the acceptor bacteria in the crosses in which the acceptor culture was in the fimbriate phase; but it had not been transmitted even to a barely detectable proportion (about 0·3 %) of the acceptor bacteria in the crosses in which the acceptor culture was in the non-fimbriate phase. Similar results were obtained regardless of whether the donor culture was in the fimbriate or the non-fimbriate phase.

After 48 hr incubation of the mixed culture the proportion of Fim+F acceptor bacteria that were colicinogenic had become still larger (93 and 95 %). By this time some of the Fim+N acceptors also had become colicinogenic, but the proportion (22 and 16 %) was much less than that in the case of the Fim+F acceptors. The late acquisition of *colI* by the originally non-fimbriate (Fim+N) acceptor bacteria could possibly have been the result of the conversion of some of these bacteria to the fimbriate phase in the later stages of culture in broth. After 48 hr a few of the cultures that originally were in the non-fimbriate phase showed a small degree of haemagglutinating activity and, therefore, must have contained a small proportion of fimbriate bacteria.

These results, indicating that the state of fimbriation of the acceptor bacteria, but not that of the donor bacteria is important in determining the frequency of transfer of *colI*, were confirmed in further experiments in which strain 1a1 as donor was crossed with Fim+F and Fim+N cultures of eleven strains of other serotypes as acceptors. The results of 10 of the 11 heterotypic crosses, 1a1 × 2a19, 2b1, 3a106, 4a1, 4a2, 4b1, 5a4, 5a12, X105, Y3, were very similar to those of the homotypic crosses (1a1 × 1a1); the results of the cross 1a1 × 2a19 are shown in Table 3. In crosses with the eleventh strain, 4b3, as acceptor the results were completely negative. Neither the fimbriate nor the non-fimbriate form of strain 4b3 acquired *colI*; this strain seemed to be absolutely incompetent as an acceptor.

Two other Fim+ strains, 2b1 and 4a1, were investigated as donors. The acceptors were streptomycin-resistant mutants of the same strains and similar mutants of strains 1a1, 2a19, 3a106, 4a2, 5a12. The crosses gave similar results to those in which strain 1a1 was donor.

Transfer of colI from genotypically non-fimbriate (Fim⁻) to genotypically fimbriate (Fim⁺) strains

Crosses were tested between the Fim⁻ strain 5a3 of *Shigella flexneri* as donor and fimbriate-phase (Fim+F) and non-fimbriate-phase (Fim+N) bacteria of twelve different Fim+ strains as acceptors. The results are given in Table 4. Just as in the crosses with Fim+ donors, the rate of transfer of *colI* from the Fim⁻ donor was high when the acceptor bacteria were in the fimbriate phase and low when they were in the non-fimbriate phase. This finding was obtained with 11 of the 12 acceptor strains. The twelfth strain, 4b3, proved to be absolutely incompetent as an acceptor, regardless of whether it was tested in the fimbriate or non-fimbriate phase.

Crosses were also made with the Fim⁻ strains 2a1 and 4c10 of *Shigella flexneri* and the Fim⁻ strain 2 of *S. sonnei* as donors. They gave similar results to those obtained with strain 5a3 as donor.

Table 4. *Transfer of colI from Shigella flexneri bacteria of Fim⁻ strain 5a3 as donors to fimbriate-phase (Fim⁺F) and non-fimbriate-phase (Fim⁺N) bacteria of 12 different Fim⁺ strains as acceptors during growth in mixed culture for 20 or 48 hr*

Crosses were made in 10 ml. broth inoculated with 10^7 donor and 10^7 acceptor bacteria and tested as described in Table 3. In each cross and at each period of incubation, between 200 and 700 acceptor-strain colonies were tested for colicinogeny (only 50 colonies in culture with acceptor 1a1 Fim⁺N at 20 hr.).

	Viable count ($\times 10^{-6}$/ml.) of the acceptor-strain bacteria in the culture in which they were				% of acceptor-strain bacteria found to be colicinogenic in the culture in which they were			
	Fim⁺F		Fim⁺N		Fim⁺F		Fim⁺N	
Acceptor strain	20 hr	48 hr	20 hr	48 hr	20 hr	48 hr	20 hr	48 hr
1a1	98	70	29	20	10	97	< 2·0	2·8
2a19	110	80	64	51	14	57	< 0·3	1·6
2b1	100	79	56	40	20	75	< 0·3	0·5
3a106	130	93	69	42	20	54	2·7	5·6
4a1	130	91	67	52	36	92	4·3	7·0
4a2	120	86	73	43	16	74	3·9	11·4
4b1	140	94	69	54	22	75	3·0	4·2
4b3	115	82	61	48	< 0·2	< 0·3	< 0·3	< 0·4
5a4	110	76	62	46	17	62	1·3	3·1
5a12	96	76	44	39	14	58	< 0·5	0·5
x105	130	92	68	47	19	54	2·1	9·1
Y3	125	88	71	49	16	44	1·2	5·0

Table 5. *Transfer of colI between different genotypically non-fimbriate strains of Shigella flexneri during growth in mixed culture for 20 or 48 hr*

Crosses were made in 10 ml. broth inoculated with 10^7 donor and 10^7 acceptor bacteria and tested as described in Table 3. The viable counts ($\times 10^{-6}$/ml.) of the acceptor-strain bacteria ranged between 25 and 120 in the different 20 hr cultures, and between 22 and 76 in the 48 hr cultures.

Cross		% of acceptor-strain bacteria found to be colicinogenic (and no. colicinogenic/no. tested) in mixed culture tested at	
Donor (Fim⁻) strain	Acceptor (Fim⁻) strain	20 hr	48 hr
2a1	1b1	< 0·9 (0/122)	< 1·1 (0/86)
	2a1	5·2 (26/502)	6·2 (20/314)
	3c2	< 0·5 (0/192)	< 0·7 (0/143)
	4c10	4·6 (6/128)	13·3 (23/172)
	5a3	< 0·3 (0/346)	< 0·3 (0/317)
4c10	1b1	< 1·0 (0/104)	< 1·2 (0/82)
	2a1	1·4 (5/354)	2·4 (6/248)
	3c2	< 0·6 (0/176)	< 1·1 (0/87)
	4c10	< 0·8 (0/123)	< 1·1 (0/92)
	5a3	< 0·3 (0/326)	< 0·5 (0/216)
5a3	1b1	< 0·5 (0/207)	1·6 (2/124)
	2a1	10·7 (42/392)	21·0 (62/295)
	3c2	< 0·4 (0/244)	2·9 (4/136)
	4c10	10·1 (19/188)	19·1 (25/131)
	5a3	6·2 (17/274)	13·2 (21/159)

Transfer of colI to genotypically non-fimbriate (Fim⁻) strains as acceptors

The results of crosses between strains that are Fim⁻ in genotype, i.e. which never form fimbriae, are given in Table 5. Three streptomycin-sensitive Fim⁻ strains carrying *colI* were tested as donors and 5 streptomycin-resistant non-colicinogenic Fim⁻ strains as acceptors. After 48 hr incubation of the mixed culture the proportion of acceptor bacteria that had acquired *colI* was, in most crosses, either small or undetectable. Nevertheless, it is noteworthy that some transfer of *colI* from Fim⁻ donor to Fim⁻ acceptor was detected in at least one cross with each of the 3 donors, and in at least one cross with each of the 5 acceptors. In 3 crosses with strain 5a3 as donor and in one with strain 2a1 as donor the percentage of colicinogenic acceptor bacteria was somewhat higher (21, 19, 13 and 13 %) than in the other Fim⁻ × Fim⁻ crosses, but it was still not nearly as high as in comparable crosses with fimbriate acceptor bacteria (e.g. 44–97 %, Table 4).

Other crosses were made between Fim⁺F bacteria of strain 1a1 as donors and the Fim⁻ strains 1b1, 2a1, 3c2, 4c10 and 5a3 as acceptors. The percentage of colicinogenic acceptor bacteria at 48 hr was never greater than 21 and in most crosses it was much less. These results confirm the relative incompetence of Fim⁻ bacteria, as compared with Fim⁺F bacteria, as acceptors of *colI*, and also show that this incompetence applies when the donor bacteria are fimbriate as well as when they are non-fimbriate.

Table 6. *Transfer of colI between donor and acceptor bacteria of Shigella flexneri Fim⁺ strain 1a1 in mixed cultures inoculated with the donor and acceptor bacteria in different proportions*

Tubes of 10 ml. broth were inoculated with the stated numbers (per tube) of donor bacteria in the fimbriate (F) phase and acceptor bacteria in the fimbriate (F) or non-fimbriate (N) phase, and were incubated for 20 or 48 hr; separate tubes were set up for each period of incubation. Between 532 and 628 acceptor-strain bacteria from each culture (tube) were tested for colicinogeny.

Cross Fimbrial phase and no. of bacteria inoculated		Viable count ($\times 10^{-6}$/ml.) of donor + acceptor bacteria (and of acceptors only) at		% of acceptor-strain bacteria found to be colicinogenic at	
Donor bacteria	Acceptor bacteria	20 hr	48 hr	20 hr	48 hr
F, 10⁷	F, 10⁴	420 (0·37)	380 (0·29)	22	69
F, 10⁷	N, 10⁴	410 (0·16)	370 (0·13)	< 0·2	2·9
F, 10⁴	F, 10⁷	390	330	0·7	34
F, 10⁴	N, 10⁷	380	320	< 0·2	< 0·2

Influence of the relative numbers of the donor and acceptor bacteria in the mixed culture

In the experiments (e.g. see Tables 3, 4) in which Fim⁺F and Fim⁺N bacteria of the same strain were compared as acceptors of *colI* in crosses with the same donor culture, the viable count of the acceptor-strain bacteria in the mixed culture after 20 or 48 hr was 2–3 times greater when the acceptors were Fim⁺F than when they were Fim⁺N. This finding agrees with the observations of Duguid & Gillies (1957) and Duguid & Wilkinson (1961) that fimbriate bacteria grow to higher concentra-

tions in aerobic static broth than do non-fimbriate bacteria; they do this apparently as a result of their ability to grow as a pellicle on the surface of the broth where they have free access to atmospheric oxygen. The question therefore arises as to whether the infrequency of transfer of *colI* to non-fimbriate acceptor bacteria was due to the growth and metabolism of these acceptor bacteria being depressed in the post-logarithmic phase of culture through their being overgrown by the fimbriate donor bacteria. This explanation is not supported by the results of mixed-culture experiments in which donor and acceptor bacteria of strain 1a1 were inoculated into broth in different proportions (Table 6). The relative numbers of the donor and acceptor bacteria did not have a very great influence on the frequency of transfer of *colI*. Indeed, in a cross in which non-fimbriate (Fim+N) acceptor bacteria in the inoculum outnumbered fimbriate (Fim+F) donor bacteria by 1000 to 1 (F, 10^4; N, 10^7), no transfer of *colI* was detected at 48 hr.

Transfer of colI–P9 from Salmonella typhimurium to Shigella flexneri

Ozeki *et al.* (1962) showed that when *Salmonella typhimurium* strain LT 2 *cysD*–36 carrying *colI–P*9 was grown overnight in mixed culture in broth with a non-colicino-genic line of the same strain, about 50 % of the latter bacteria acquired *colI–P*9. We made tests with strain LT 2 *cysD*–36 *colI–P*9, which is Fim+, to determine whether it was as efficient in donating *colI–P*9 to *Shigella flexneri* as it was to *S. typhimurium*, and whether the frequency of transfer of *colI–P*9 was influenced by the state of fimbriation of the acceptor bacilli in the same way as was the transfer of *colI* from *S. flexneri* donors. *S. typhimurium* LT 2 *cysD*–36 *colI–P*9 was crossed with 5 Fim⁻ and 12 Fim+ strains of *S. flexneri* as acceptors; the Fim+ acceptors were tested both in the fimbriate and the non-fimbriate phase. Mixed cultures were grown in 10 ml. broth from inocula of 10^7 donor and 10^7 acceptor bacteria. The proportions of acceptor-strain bacteria found to be colicinogenic after 20 and 48 hr were invariably low in the crosses with Fim⁻ acceptors (under 2·5 %) and Fim+N acceptors (under 7 %), but were high (e.g. 40 %) in those with Fim+F acceptors. These results of crosses with the *S. typhimurium* donor were very similar to the results of the crosses with *S. flexneri* donors. A minor difference was that in the crosses with *S. typhimurium* the proportion of colicinogenic acceptor bacteria did not continue to increase when incubation of the mixed culture was prolonged from 20 to 48 hr, but instead became somewhat smaller again. The absence of continued transfer between 20 and 48 hr may have been due to the fact that the *S. flexneri* acceptor bacteria became very much overgrown by the *S. typhimurium* donor bacteria. At 48 hr the viable counts ($\times 10^6$)/ml. in the mixed culture were: *S. typhimurium* 570 and *S. flexneri* 5·7 when *S. flexneri* was Fim+F, and *S. typhimurium* 570 and *S. flexneri* 2·6 when *S. flexneri* was Fim+N.

Kinetics of transfer of colI

The frequency of transfer of *colI* was observed after different periods of incubation from 2 to 72 hr in crosses between Fim+F bacilli of *Shigella flexneri* strain 1a1 as donors and Fim+F bacteria of *S. flexneri* strains 1a1 and 2a19 as acceptors. A separate tube of mixed broth culture was set up for examination after each period of incubation and about 500 acceptor-strain colonies in platings from each culture were

examined for colicinogeny. The results of the experiment with strain 1a1 as acceptor are given in Table 7; almost identical results were obtained in a similar experiment with strain 2a19.

No transfer of *colI* was detected after the first 2 hr of incubation, but after 4 hr a small proportion (0·3 %) of acceptor bacteria possessed *colI* and the proportion of acceptors with *colI* increased slowly up to 12 hr (4 %) and then more rapidly up to 24 hr (30 %). By 48 hr, almost all (97 %) of the acceptor bacteria were colicinogenic. The transfer of *colI* thus took place mainly in the period of slow post-logarithmic growth, which began after the first 6 hr of incubation. Most transfers took place after the 12th hr, by which time a pellicle had developed on the surface of the culture.

Table 7. *Kinetics of transfer of colI between fimbriate-phase donor and acceptor bacteria of Shigella flexneri strain 1a1 during growth in mixed culture at 37°*

Nine tubes of 10 ml. broth were each inoculated with 10^7 Fim$^+$F donor bacteria and 10^7 Fim$^+$F acceptor bacteria and incubated under aerobic static conditions at 37° for different periods up to 72 hr. After incubation, the culture was homogenized and tested for the viable count of acceptor-strain bacteria and the proportion of acceptor-strain bacteria which were colicinogenic. A separate tube of mixed culture was used for each period of incubation so that it and any pellicle it formed were undisturbed until the time of testing.

Period of incubation of mixed culture (hr)	Viable count of acceptor-strain bacteria ($\times 10^{-6}$/ml.)	% of acceptor-strain bacteria found to be colicinogenic (and no. colicinogenic/no. tested)
0	1·0	.
2	5·6	< 0·2 (0/528)
4	68	0·3 (2/683)
6	130	0·4 (2/548)
8	180	2·0 (13/647)
10	190	3·2 (18/571)
12	190	4·1 (23/562)
24	180	30 (187/624)
48	120	97 (585/603)
72	89	100 (442/442)

There seem to be two possible explanations for the very low frequency of transfer of *colI* in the early hours of incubation of the mixed cultures. (1) The initial concentration of the inoculated bacteria (donors and acceptors each about 10^6/ml.) may have been so low that collisions and contacts between bacteria, and thus opportunities for transfer of *colI*, were rare until the numbers of bacteria had increased as a result of several hours' growth. Against this explanation, however, should be noted the fact that transfer was still relatively infrequent throughout the period between the 4th and 12th hours of incubation, i.e. in a period when the number of viable bacteria was nearly maximal. A possible reason for the finding that the majority of transfers took place after 12 hr is that the best opportunities for contact and transfer of *colI* between bacteria existed in the pellicle of bacteria which developed on the surface of the broth after about the 10th hour of incubation and in which most of the slow post-logarithmic growth of bacteria presumably took place.

(2) Only a small proportion of the bacteria of the donor cultures may have been competent to transmit the *colI* factor. Ozeki *et al.* (1962) found that the low fre-

quency of transfer of *colI–P*9 during the first 6 hr of incubation of mixed cultures of donor and acceptor stocks of *Salmonella typhimurium* LT2 *cysD*–36 was due to the fact that only about 0·02 % of the bacteria in the donor cultures were competent to transmit the *colI–P*9 factor. They explained the high rate of transfer after pro-longed (20 hr) incubation as being the result of an 'epidemic' spread of *colI–P*9 which took place through the acceptor population during the period of slow post-logarithmic growth. Apparently a large proportion of the freshly infected acceptor bacteria become competent donors, able to conjugate with other acceptor bacteria and pass *colI–P*9 into them. Stocker *et al.* (1963) showed that 50 % of the *S. typhi-murium* bacteria which had freshly acquired *colI–P*9 became competent donors and were able to pass the factor into acceptor bacteria with high frequency. Pre-parations with a high proportion of competent donor bacteria were named HFC preparations (high-frequency colicinogeny-transferring); when mixed with acceptor bacteria at concentrations of more than 10^8 bacteria/ml., they transmitted *colI–P*9 to about 50 % of the acceptor bacteria in 1 hr.

Attempt to prepare an HFC culture of Shigella flexneri

The technique of Stocker *et al.* (1963) was used. About 5×10^5 Fim⁺F bacteria of streptomycin-sensitive *Shigella flexneri* strain 1a1 carrying *colI* or *colI–P*9 as original donors and about 10^7 Fim⁺F non-colicinogenic streptomycin–sensitive 1a1 bacteria as intermediate donors were inoculated into 10 ml. broth and the mixed culture incubated for 18 hr. The culture was then diluted tenfold with fresh broth and incubated for a further 2 hr. After the first 18 hr of incubation the proportion of colicinogenic bacteria was, in different experiments, between 17 and 33 %, and after dilution and secondary incubation for 2 hr it was only about half as much (9–21 %). These values are much lower than those obtained by Stocker *et al.* with *Salmonella typhimurium*, namely 30–70 % after the first 18 hr and 50–90 % after secondary 2 hr incubation. Thus *S. flexneri* did not behave in the same manner as *S. typhimurium* and our attempt to prepare an HFC culture was not pursued. A possible reason for the different behaviour of *S. flexneri* may lie in its non-motility, since Stocker *et al.* found that motility was important in the development of HFC preparations of *S. typhimurium*. They found that when the intermediate (non-coli-cinogenic) strain was non-motile, the proportion of the bacteria that acquired coli-cinogeny after primary incubation for 18 hr and secondary incubation for 2 hr was less than 1 %, whereas when the intermediate strain was motile the proportion was about 70 %.

'Epidemic' spread of colI within the acceptor population

Although we failed to demonstrate by the production of an HFC culture that *Shigella flexneri* organisms which have been freshly infected with *colI* are more effective in transmitting *colI* than the bacteria of a long-established colicinogenic culture, it still seemed possible that such bacteria, like comparable *Salmonella typhimurium* organisms, may be exceptionally competent as donors. If they are specially competent, then it is likely that the majority of the acceptor bacteria that become colicinogenic during 48 hr in a mixed culture do so as a result of the *colI* factor spreading 'epidemically' from one acceptor bacterium to another, and not by acquiring the factor directly from the bacteria of the donor strain. An experiment

was made to determine whether such an 'epidemic' spread of *colI* took place within the acceptor population. The method of the experiment involved adding streptomycin (1 mg./ml.), to a mixed culture to kill the streptomycin-sensitive donor bacteria after the culture had been incubated for only 8 hr and when only a few bacteria of the streptomycin-resistant acceptor strain had acquired *colI*; the culture was then re-incubated for the remainder of a period of 20 or 48 hr. It was found that the proportion of acceptor bacteria possessing *colI* increased very greatly during the secondary incubation and, since all the donor strain bacteria were then dead, the increase must have been the result of an 'epidemic' spread of *colI* taking place through the population of acceptor-strain bacteria.

Table 8. *Transfer of colI between donor and acceptor Shigella flexneri organisms grown in mixed cultures with and without the addition of streptomycin after the first 8 hr*

For each cross, 4 tubes of 10 ml. broth were inoculated with 10^6 streptomycin-sensitive donor bacteria and 10^7 streptomycin-resistant acceptor bacteria per tube. After incubation at 37° for 8 hr, each culture was mixed until homogeneous and samples were taken from it for estimation of the viable count of acceptor-strain bacteria and the proportion that were colicinogenic. Streptomycin (1 mg./ml.) was added to two of the cultures and all four were then reincubated. A streptomycin-treated and an untreated culture were examined after a total of 20 hr incubation and the other two cultures at 48 hr.

Cross			Viable count ($\times 10^{-6}$/ml.) of acceptor-strain bacteria at			% of acceptor-strain bacteria found to be colicinogenic at		
Donor bacteria	Acceptor bacteria	Treatment at 8 hr	8 hr*	20 hr	48 hr	8 hr†	20 hr	48 hr
1a1 Fim⁻F	1a1 Fim⁺F	Untreated	320	360	340	0·44	30	76
		Streptomycin	320	355	345	0·45	0·37	62
1a1 Fim⁺F	2a1 Fim⁻	Untreated	290	305	215	0·28	12	24
		Streptomycin	290	310	220	0·29	0·24	0·47
5a3 Fim⁻	1a1 Fim⁺F	Untreated	335	345	335	0·69	37	87
		Streptomycin	335	340	260	0·34	1·8	69

* Results are averages for the four tubes of the cross. † Results are averages for the two tubes that were later examined at 20 and 48 hr.

Table 8 shows the results of experiments made in this way on three crosses of *Shigella flexneri* strains: (1) donor Fim⁺F × acceptor Fim⁺F, (2) donor Fim⁺F × acceptor Fim⁻; (3) donor Fim⁻ × acceptor Fim⁺F. In each of the crosses a small proportion (0·28–0·69 %) of the acceptor bacteria had acquired *colI* by the end of the first 8 hr of incubation. In the cultures with Fim⁺F acceptors that were treated with streptomycin at 8 hr and then reincubated until 48 hr, the proportion of acceptor bacteria possessing *colI* rose to values (62 and 69 %) nearly as high as those (76 and 87 %) in the control cultures without streptomycin. On the other hand, the proportion of colicinogenic acceptor bacteria remained very low (under 0·5 %) in the streptomycin-treated cultures in which the acceptor was Fim⁻. Thus it appeared that *colI* spread epidemically through the population of acceptor bacteria when these were fimbriate (Fim⁺F), but did not do so when they were non-fimbriate (Fim⁻). The continuing acquisition of *colI* by Fim⁺F acceptor bacteria after the donor-strain bacteria had been killed with streptomycin must have been due to an epidemic

spread of *colI* that originated from the few acceptor bacteria that had acquired *colI* from the donor-strain bacteria before the addition of streptomycin. It may be assumed that the acceptor bacteria did not acquire *colI* from the killed donor bacteria, since it was found that when streptomycin was added at 2 hr instead of 8 hr, i.e. before any acceptor bacteria had become colicinogenic, none of the acceptor bacteria became colicinogenic during secondary incubation for 48 hr.

An interesting difference in the results between the streptomycin-treated and the untreated crosses with Fim⁺F acceptors (Table 8) is seen in the finding that in the streptomycin-treated cultures the increase in the proportion of colicinogenic acceptor bacteria was delayed until after the 20th hour of incubation, whereas in the untreated cultures it was already very great at 20 hr (compare 0·37 with 30 %). This finding seems to indicate that in the untreated cultures the large increase in colicinogenic acceptors that took place between 8 and 20 hr was mainly due to transfer of *colI* from the donor-strain bacteria to the acceptor-strain bacteria, and not to epidemic spread among the acceptors. It may perhaps also be concluded that the proportion of the donor-strain bacteria in the culture at 8 hr that were 'competent donors' of *colI* was at least much greater than about 0·5 %, which was the proportion of the tenfold larger population of acceptor bacteria that by 8 hr had become colicinogenic. In contrast, Ozeki *et al.* (1962) found that only about 0·02 % of the bacteria in an established colicinogenic stock of *Salmonella typhimurium* strain LT2 were 'competent donors'. However, these various conclusion are valid only on the assumption that the streptomycin added to our cultures had no effect other than that of killing the donor-strain bacteria.

Control tests indicated that the increase of colicinogenic bacteria in the streptomycin-treated cultures was not due to the emergence, survival and multiplication of streptomycin-resistant mutants of the colicinogenic donor strain. The rates of mutation of strains 1a1 and 5a3 to resistance to 1 mg. streptomycin/ml. are very low, less than 1 per 10^{11} bacteria, and the number of viable donor-strain bacteria in the mixed cultures at 8 hr cannot have exceeded about 10^9/10 ml. tube. Fifty pure cultures of the donor strain 1a1 and 50 of the donor strain 5a3 were grown for 8 hr in tubes of 10 ml. broth inoculated with about 10^6 bacteria; streptomycin was then added and incubation continued until 48 hr. None of these control cultures yielded a growth of streptomycin-resistant mutants. Serological agglutination tests were made on 400 streptomycin-resistant colicinogenic colonies obtained in platings grown from the streptomycin-treated mixed cultures of 5a3 donor and 1a1 acceptor bacteria after they had been incubated for 20 and 48 hr (Table 8) and on another 400 such colonies from the corresponding cultures grown without the addition of streptomycin; all 800 colonies were found to be of the acceptor (1a) serotype. This finding confirms that the streptomycin-resistant colicinogenic organisms were not donor strain (5a) bacteria which had acquired resistance to streptomycin either by spontaneous mutation or by transfer of a resistance factor from the acceptor bacteria.

Pellicle formation and the transmission of colI

In cultures of *Shigella flexneri* in aerobic static broth the development of fimbriae is associated with the formation on the surface of the broth of a thin pellicle composed of aggregated bacteria. In cultures inoculated with fimbriate-phase organisms, e.g. Fim⁺F 1a1 bacteria, the pellicle appears after about 10 hr of incubation

(Duguid & Wilkinson, 1961). Since, in our crosses with Fim⁺F acceptors, the main increase in the proportion of colicinogenic acceptor bacteria took place only in the period of incubation after the first 10–12 hr (e.g. see Table 7), it seemed possible that the conjugation of bacteria and the transfer of *colI* were effected principally in the pellicle. To investigate this possibility the following experiment was done.

A tube of 10 ml. broth was inoculated with 10^7 Fim⁺F donor bacteria and 10^7 Fim⁺F acceptor bacteria and incubated under aerobic static conditions for 8 hr. The culture was then homogenized and examined for colicinogenic acceptor bacteria; the proportion of these was found to be about 1 %. The culture then was re-incubated for 12 hr, but during this second period of incubation it was gently agitated at intervals of 1 hr. The intermittent agitation was sufficient to prevent the formation of a surface pellicle, but it could not have effectively aerated the culture or have interfered mechanically with the conjugation of bacteria, since the culture was static for the greater part of the 12 hr period. After the total of 20 hr of incubation, the proportion of colicinogenic acceptor bacteria was found to have increased only to 7 %. By contrast, the proportion in an unagitated control culture increased from 1 % at 8 hr to 34 % at 20 hr. Both the agitated and control cultures showed strong haemagglutinating activity, indicating that they were richly fimbriate. When the agitated culture was left undisturbed for a further 28 hr at 37°, a surface pellicle appeared and the proportion of colicinogenic acceptor bacteria increased to 95 %. These findings seem to indicate that the formation of a surface pellicle facilitates the transmission of *colI*.

Influence of aeration of the mixed culture on the transfer of colI

When broth cultures (2 ml. broth in a 20 ml. bottle) inoculated with Fim⁺F donor and Fim⁻F acceptor bacteria of strain 1a1 were agitated continuously in a rotating machine while being incubated for 20 hr, the proportion of colicinogenic acceptor bacteria did not increase above 2 %. The cultures did not form a pellicle, although they were moderately richly fimbriate. The poor transfer of *colI* may, as in the previous experiment, have been due to to the effect of agitation in preventing pellicle formation. However, an additional factor may have played a part in this experiment in which agitation was continuous and aeration therefore efficient. In the continuously aerated culture, the growth proceeds rapidly and gives a maximum yield of bacteria within about 6 hr. After this time it ceases abruptly, probably as a result of exhaustion of the oxidizable energy-yielding nutrients in the medium. Ozeki *et al.* (1962), who found that transfer of *colI–P*9 in mixed cultures of *Salmonella typhimurium* grown in aerobic static broth took place mainly during the prolonged phase of slow post-logarithmic growth, and that aeration by continuous shaking prevented transfer of the *colI* factor, considered that the inhibition produced by aeration might be due to the effect of aeration in eliminating the phase of post-logarithmic growth.

Transfer of colI in mixtures of separately grown cultures of donor and acceptor strains

In all the experiments described above the mixed cultures were grown from relatively small inocula, e.g. about 10^7 donor and 10^7 acceptor bacteria, and this procedure allowed the bacteria to pass through a substantial period of rapid logarithmic

growth. Other experiments were made under different conditions. The donor and acceptor strains were first grown separately for 24 hr in tubes of aerobic static broth. These post-logarithmic cultures, which contained $1-4 \times 10^8$ viable bacteria/ ml., were mixed together in a tube, 5 ml. with 5 ml., and the mixture was incubated for a period of 8, 20 or 48 hr without the addition of any fresh broth. This procedure caused the donor and acceptor bacteria to be mixed in high concentrations from the outset and it permitted only a slow and limited growth in the mixture; the total count (turbidity) increased about twofold in 48 hr.

Table 9. *Transfer of colI between 24 hr fimbriate-phase (F) and non-fimbriate-phase (N) broth cultures of Shigella flexneri strain 1a1 after mixture and incubation without the addition of fresh broth*

For each cross, 3 tubes were set up containing a homogeneous mixture of 5 ml. of a 24 hr broth culture of the donor bacteria and 5 ml. of a 24 hr broth culture of the acceptor bacteria. The mixtures were incubated under aerobic static conditions at 37° for 8, 20 or 48 hr before harvesting and testing.

Cross		Viable count ($\times 10^{-6}$/ml.) of acceptor-strain bacteria at			% of acceptor-strain bacteria found to be colicinogenic at		
Donor bacteria in phase	Acceptor bacteria in phase	8 hr	20 hr	48 hr	8 hr	20 hr	48 hr
F	F	120	100	75	1·2	66	91
F	N	51	42	31	< 0·4	< 0·4	22
N	F	140	120	100	0·9	50	92
N	N	54	38	32	< 0·3	< 0·5	4·8

Table 9 shows the results of crosses between Fim⁺F and Fim⁺N cultures of *Shigella flexneri* strain 1a1. Separate tubes of each mixture were put up for examination after 8, 20 and 48 hr. There was little transfer of *colI* during the first 8 hr after mixing, although throughout this period the donor and acceptor bacteria were exposed to each other in high concentrations and under conditions favourable to slow post-logarithmic growth. A few colicinogenic acceptor bacteria were found at 8 hr in the mixtures in which the acceptors were in the fimbriate phase, but the proportion of these bacteria (about 1 %) was no higher than it was in comparable cultures grown for 8 hr from small inocula. The delay in transfer of *colI* may have been due to the time taken for re-establishment of a surface pellicle after the mixing of the cultures.

In the crosses in which the acceptor was Fim⁺F the proportion of colicinogenic acceptor bacteria increased sharply after the first 8 hr and reached fairly high values (50 and 66 %) at 20 hr, and very high values (91 and 92 %) at 48 hr. The crosses in which the acceptor was Fim⁺N did not yield colicinogenic acceptor bacilli until 48 hr and then did so only in a proportion much lower (4·8 and 22 %) than that in the crosses with Fim⁺F acceptors. These results are very like the results obtained for mixed cultures grown from small inocula.

When the donor and acceptor cultures were grown for several (up to 7) days before they were mixed, the proportions of colicinogenic acceptors after 20 and 48 hr of incubation of the mixture were almost as high as those in the mixtures of 24 hr cultures. When, however, the parent cultures were still older the rate of transfer of

colI was decreased. Thus, in a cross of donor 5a3 Fim⁻ with acceptor 1a1 Fim⁻F, the percentages of colicinogenic acceptor bacteria at 20 and 48 hr were 36 and 97 when the age of the parent cultures before mixture was 24 hr, 75 and 98 when the age was 48 hr, 36 and 95 when it was 5 days, 20 and 76 when it was 10 days, 1 and 27 when it was 15 days, and < 0·2 and 8 when it was 20 days. The diminishing rate of transfer in the mixtures of the older cultures may have been due to the diminishing viability of the bacteria in them.

In the experiments just described, the parent cultures and the mixture of them were incubated under aerobic static conditions, which permitted them to form a pellicle and undergo a prolonged phase of slow post-logarithmic growth. When, instead, the separate parent cultures (donor 1a1 Fim⁺F and acceptor 1a1 Fim⁺F) were grown for 24 hr with continuous agitation and aeration, no detectable transfer of *colI* took place when the mixture was incubated under aerobic static conditions for 48 hr. Ozeki *et al.* (1962) obtained a similar result with aerated cultures of *Salmonella typhimurium* and they attributed the absence of transmission to the energy sources in the medium being exhausted before the cultures were mixed.

Transfer of colI in defined medium

A high rate of transmission of *colI* was demonstrated in mixed cultures of donor 5a3 Fim⁻ and acceptor 1a1 Fim⁺F bacteria grown in liquid glucose ammonium salts medium supplemented with nicotinic acid. Strain 1a1 formed a delicate pellicle on the surface of this medium and the culture gave a positive haemagglutination reaction, indicating that it was fimbriate. Separate cultures of the donor and acceptor bacteria were grown in the defined medium to provide the inocula for the mixed cultures. The mixed cultures, which were inoculated with 10^7 donor and 10^7 acceptor bacteria, were grown in 10 ml. defined medium under aerobic static conditions for 48 hr. At the end of this period, in different experiments, between 37 and 58 % of the acceptor bacteria were colicinogenic. When the bacteria from donor and acceptor cultures which had been grown for 48 hr in defined medium were washed twice in saline, resuspended in equal proportion and to their original concentration in fresh defined medium, and then incubated for another 48 hr, the proportion of the acceptor bacteria, that became colicinogenic ranged from 46 to 74 %. When such mixtures of washed donor and acceptor bacteria (> 10^8 viable bacteria/ml.) were incubated in defined medium that lacked either glucose or nicotinic acid, both of which were essential for growth, there was little or no transfer of *colI* (< 1 %).

DISCUSSION

The colicinogenic factor *colI* was found to be transmitted between the bacteria of different strains and serotypes of *Shigella flexneri* when these bacteria were grown together in mixed culture in aerobic static broth for 20 or 48 hr at 37°. Transmission, though at a low rate, was observed in crosses in which both the donor and acceptor organisms were genotypically non-fimbriate (Fim⁻) and it is therefore clear that the presence of fimbriae is not *essential* for transmission. By 'fimbriae', in this context, we mean the ordinarily numerous kind of non-flagellar filaments that are easily demonstrable by electron microscopy and haemagglutination tests. Scanty 'F fimbriae' ('F pili') of the kind demonstrated by Crawford & Gesteland (1964)

and Brinton, Gemski & Carnahan (1964) in *Escherichia coli* and thought by the latter authors to serve as an organ of conjugation in enterobacteria, would not have been detected by us if they had been present in the strains of *S. flexneri* that we have designated Fim⁻.

A *high rate* of transmission of *colI* was obtained only in those crosses in which the acceptor *Shigella flexneri* organisms were both genotypically and phenotypically fimbriate (Fim⁺F). Under these conditions, and regardless of whether the donor organisms were fimbriate or non-fimbriate, usually between 50 and 95 % of the acceptor bacteria became colicinogenic during growth for 48 hr in mixed culture. When the acceptor bacteria were either genotypically non-fimbriate (Fim⁻) or genotypically fimbriate but in the non-fimbriate phase (Fim⁺N), the proportion of them which became colicinogenic was usually only between 0·2 and 20 %. The expression of fimbriation in the acceptor bacteria thus had the effect of promoting a high rate of transmission, whereas the state of fimbriation in the donor bacteria was without effect.

In many respects the conditions and course of transmission of *colI* observed in our experiments with fimbriate *Shigella flexneri* organisms resembled those observed by Ozeki *et al.* (1962) and Stocker *et al.* (1963) in similar experiments with the fimbriate strain LT 2 *cysD*–36 of *Salmonella typhimurium*. Thus, in both species the transmission of *colI* factor in mixed cultures in aerobic static broth took place mainly in the period of slow post-logarithmic growth, and in the case of both species there was evidence that this transmission involved an extensive 'epidemic' spread of the *colI* factor within the acceptor population, i.e. from freshly infected acceptor bacteria, to other acceptor bacteria. In both species, too, transmission was almost completely absent when the mixed cultures were aerated by continuous agitation. A difference in behaviour between the species was noted in our failure to obtain an HFC preparation of *S. flexneri* that contained a high proportion of colicinogenic organisms after 2 hr of secondary incubation of the mixture of the original donor and the intermediate strain. Since, however, Stocker *et al.* also failed to obtain a high proportion of colicinogenic organisms in such cultures when they used a non-motile mutant of *S. typhimurium* as the intermediate strain, our poor result with non-motile *S. flexneri* is not surprising. Ozeki *et al.* and Stocker *et al.* did not use any Fim⁻ strain or any proven non-fimbriate-phase culture of *S. typhimurium*, so that it is not known whether the presence of fimbriae in the acceptor bacteria promotes transmission of *colI* in *S. typhimurium* in the same way as it does in *S. flexneri*.

The reason why transmission of *colI* is promoted by the presence of fimbriae in *Shigella flexneri* acceptors has not been determined. The finding that fimbriae (of the ordinarily demonstrable kind) are not essential for transmission makes it seem unlikely that they act as specific organs for transfer and intromission of the *colI* factor. It may be, however, that they facilitate the adhesion and conjugation of organisms as a necessary preliminary to transfer. There is good evidence to show that fimbriae have the effect of causing bacteria to adhere to various kinds of animal, plant and fungal cells, but none to show that they cause bacteria which are submerged in a liquid medium to adhere to one another. On the other hand, it has been found that the presence of fimbriae in *S. flexneri* organisms causes these, when cultured in aerobic static broth, to grow aggregated together in large numbers in a

pellicle that appears on the surface of the broth after incubation for about 10 hr (Duguid & Gillies, 1957); some Fim⁻ strains also may form a pellicle, but they do this at a later stage in culture, e.g. after 2–3 days. It may be supposed that the aggregation of fimbriate bacteria in a pellicle increases the opportunity for conjugation and transfer of *colI* between them. That pellicle formation is indeed responsible for the high rate of transfer obtained in crosses with fimbriate acceptors is suggested by our finding that relatively few acceptor bacteria became colicinogenic in mixed cultures in which pellicle formation was prevented by gentle shaking at intervals of 1 hr.

Another way in which fimbriation may help to promote transfer of *colI* is by its increasing, through the effect of pellicle formation, the amount of growth during the post-logarithmic phase in the mixed culture. Duguid & Wilkinson (1961, see fig. 3) found that in fimbriate-phase cultures of *Shigella flexneri* strain 1a1 in aerobic static broth the total count of bacteria increased sevenfold between the 6th hr of incubation, when logarithmic growth ended, and the 48 hr. In non-fimbriate-phase cultures the corresponding increase was only twofold. Since the transmission of *colI* in mixed cultures takes place mainly in the post-logarithmic period, it is understandable that the better growth given in this period by fimbriate as compared with non-fimbriate acceptor bacteria, may favour a higher rate of acquisition of *colI*.

If, as has been suggested, fimbriae promote transfer of *colI* through their effects in causing the aggregation and conjugation of bacteria in a pellicle and in increasing the amount of post-logarithmic growth, it may be asked why it is only the acceptor's fimbriation status that is important and why high rates of transfer are obtained with non-fimbriate donors. A possible explanation of the relative unimportance of the fimbriation status of the donor is afforded if we accept the conclusion, suggested by the experiments in which donors were killed by addition of streptomycin after 8 hr, that a high rate of transfer is the result of an extensive epidemic spread of *colI* among the acceptor bacteria. In a cross of non-fimbriate donor bacteria with fimbriate acceptor bacteria a few acceptor bacteria must first acquire *colI* from the non-fimbriate donors. Subsequently, *colI* may be transmitted freely from one fimbriate acceptor bacterium to another during the prolonged period of their post-logarithmic growth in the surface pellicle. This spread of *colI* would be little affected by the failure of the original non-fimbriate donor bacteria to participate equally in the post-logarithmic and pelliculate growth.

REFERENCES

ABBOTT, J. D. & SHANNON, R. (1958). A method for typing *Shigella sonnei* using colicine production as a marker. *J. clin. Path* **11**, 71.

BRINTON, C. C., GEMSKI, P. & CARNAHAN, J. (1964). A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K12 and its role in chromosome transfer. *Proc. natn. Acad. Sci. U.S.A.* **52**, 776.

CRAWFORD, E. M. & GESTELAND, R. F. (1964). The adsorption of bacteriophage R-17. *Virology* **22**, 165.

DUGUID, J. P. & GILLIES, R. R. (1957). Fimbriae and adhesive properties in dysentery bacilli. *J. Path. Bact.* **74**, 397.

DUGUID, J. P. & WILKINSON, J. F. (1961). Environmentally induced changes in bacterial morphology. *Symp. Soc. gen. Microbiol.* **11**, 69.

DUGUID, J. P., SMITH, I. W., DEMPSTER, G. & EDMUNDS, P. N. (1955). Non-flagellar filamentous appendages ('fimbriae') and haemagglutinating activity in *Bacterium coli. J. Path. Bact.* **70**, 335.

FREDERICQ, P. (1954). Transduction génetique des propriétés colicinogénes chez *Escherichia coli* et *Shigella sonnei. Séanc. C. r. Soc. Biol.* **148**, 399.

FREDERICQ, P. (1957). Colicins. *A. Rev. Microbiol.* **11**, 7.

FREDERICQ, P. (1963). On the nature of colicinogenic factors: a review. *J. theoret. Biol.* **4**, 159.

LEDERBERG, J. (1952). Cell genetics and hereditary symbiosis. *Physiol. Rev.* **32**, 403.

MULCZYK, M. (1960). Studies on antigenic variability of *Shigella flexneri* 1b. *Archwm. Immun. Terap. doświad,* **8**, 1.

OZEKI, H., HOWARTH, S. L. & CLOWES, R. C. (1961). Colicine factors as fertility factors in bacteria. *Nature, Lond.* **190**, 986.

OZEKI, H., STOCKER, B. A. D. & SMITH, S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *J. gen. Microbiol.* **28**, 671.

STOCKER, B. A. D., SMITH, S. M. & OZEKI, H. (1963). High infectivity of *Salmonella typhimurium* newly infected by the *colI* factor. *J. gen. Microbiol.* **30**, 201.

Effect of Ferricyanide on Energy Production by *Escherichia coli*

By LYGERI P. HADJIPETROU, T. GRAY-YOUNG and M. D. LILLY

Department of Chemical Engineering (Biochemical Engineering Section), University College London

SUMMARY

The reduction of ferricyanide by resting and actively growing *Escherichia coli* was studied. Under anaerobic conditions ferricyanide acted as a hydrogen acceptor for the complete oxidation of glucose and TCA cycle intermediates. However, reduction of ferricyanide was not coupled to ATP formation. The anaerobic molar growth yield from glucose in the presence of ferricyanide was even less than in its absence. Ferricyanide repressed the synthesis of formate hydrogenylase.

INTRODUCTION

The role of oxygen as a hydrogen acceptor in microbial metabolism has been extensively studied, but little is known about various other compounds, often called artificial hydrogen acceptors, which can replace oxygen during the growth and respiration of micro-organisms. Nason (1962) discussed the functioning of nitrate, nitrite and hydroxylamine as artificial hydrogen acceptors during metabolism. Nitrate can support anaerobic growth of *Escherichia coli* with succinate, fumarate or acetate as sole carbon and energy source (Quastel, Stephenson & Whetham, 1925; Egami, Ishimoto & Taniguchi, 1961; Hadjipetrou, unpublished). With *Aerobacter aerogenes* the anaerobic growth yield increases in the presence of nitrate with glucose as carbon and energy source, but acetate accumulates, indicating that the TCA cycle does not operate with nitrate as hydrogen acceptor (Hadjipetrou & Stouthamer, 1965). The use of sulphate as a hydrogen acceptor by the sulphate-reducing bacteria has been reviewed by Senez (1962) and Postgate (1965).

In this laboratory various types of biochemical fuel cell are being studied (Gray-Young, Hadjipetrou & Lilly, 1966). The use of artificial hydrogen acceptors in microbiological redox fuel cells is being investigated and the work described here was done to clarify the role of ferricyanide as a hydrogen acceptor for *Escherichia coli*. Ferricyanide and several redox dyes have often been used as hydrogen ac-ceptors in the study of oxidases and dehydrogenases in animal tissues. Diaphorases which catalyse the oxidation of NADH by various dyes and ferricyanide are present in several bacteria (Dolin, 1961).

METHODS

Organism. The organism used throughout this work was a culture of *Escherichia coli* K 12 which was stored on peptone liver-digest agar slopes at 4°.

Growth. For the experiments with washed organisms, *Escherichia coli* was grown overnight in either peptone liver-digest glucose or peptone liver-digest succinate media on a reciprocating shaker at 37°. The bacteria were collected by centrifugation, washed twice with 0·06 M-potassium phosphate buffer (pH 7·0) containing mM-MgCl$_2$ and resuspended in this buffer solution to give a concentration of bacteria equivalent to 8 mg. dry wt./ml.

For the growth experiments the required amount of the carbon energy source being studied was added either to the peptone liver-digest medium or to a liver-digest medium also containing (g./l.); KH$_2$PO$_4$, 5·4; MgSO$_4$, 7H$_2$O, 0·4; (NH$_4$)$_2$SO$_4$, 1·2; NaCl, 0·5; adjusted to pH 7·0 with KOH. Growth was followed by measurement of the increase in the extinction of the culture (E$_{660}$) in a Unicam SP 600 spectrophotometer. A standard curve was used to convert the extinction readings to equiv. dry wt. bacteria/ml.

Determination of the reduction of ferricyanide. The reduction of ferricyanide was followed potentiometrically in reaction vessels, immersed in a water bath at 37°, containing: potassium ferricyanide and ferrocyanide in various concentrations; the substrate carbon and energy source; bacterial suspension; phosphate buffer to 12·5 ml. final volume; pH 7·0. The potentiometric assay was carried out by measuring the potential difference between a platinum electrode immersed in the reaction vessel and a similar electrode immersed in a vessel containing equal concentrations of potassium ferricyanide and ferrocyanide in the buffer solution. The two vessels were connected by a KCl-agar bridge. The potential differences were recorded on a multipoint model DE potentiometric recorder (Cambridge Instrument Co. Ltd., London). This potential difference is related to the ratio ferricyanide: ferrocyanide concentrations; ferricyanide concentrations in the reaction mixtures were calculated from these values by reference to a standard curve. Oxygen was excluded from the reaction mixture by flushing with O$_2$-free nitrogen before the start and during the experiments. No ferricyanide reduction by any of the substrates was detected in the absence of added bacteria.

Measurement of gas uptake and evolution. The oxygen uptake, carbon dioxide and hydrogen evolution were measured manometrically in Warburg flasks (a liquid volume of 2·5 ml.) and the same concentrations of components and conditions as for the potentiometric assay method.

Chemical determinations. Samples were taken at intervals from the reaction vessels. Zinc sulphate was added to precipitate ferricyanide, ferrocyanide and the bacteria. After centrifugation the clear supernatant fluid was adjusted to about pH 8 with 50 % (w/v) KOH to precipitate the remaining zinc sulphate and traces of ferric ions which interfere in the determination of glucose by the method of Mark (1959). Steam-volatile acids were separated by steam distillation in a Markham still and estimated as described by Neish (1952). Formate was also calculated from the hydrogen evolved by samples of the steam distillates to which suspensions of washed *Escherichia coli* containing formate hydrogenylase had been added. Pyruvate and α-oxoglutarate were determined by the method of Friedemann & Haugen (1943), and ethanol by the methods described by Neish (1952).

RESULTS

The rate of ferricyanide reduction by suspensions of washed *Escherichia coli* increased with initial ferricyanide concentration, reaching a maximum at about 30 mM-ferricyanide (Fig. 1). However, above a ferricyanide concentration of 100 mM the reduction rate decreased to a new and constant value. The total carbon dioxide evolved and ferricyanide reduced during these experiments are also shown in Fig. 1. All the ferricyanide was reduced at low ferricyanide concentrations, reaching a maximum when the total amount of ferricyanide was high enough to allow all the glucose to be consumed. At concentrations above this value the total ferricyanide reduced decreases. The curve showing the total evolution of carbon dioxide follows the same pattern. In the first part of the curve the carbon dioxide is related to the amount of ferricyanide present, as formate dehydrogenase will only function in the presence of hydrogen acceptors (Linnane & Wrigley, 1963). Thus when all the ferricyanide was reduced no more carbon dioxide was evolved and formate accumulated. Since the bacteria used in these experiments had been grown in well-aerated conditions with succinate as energy source, they did not contain formate hydrogenlyase and therefore in no case was hydrogen evolved. At high ferricyanide concentrations the total carbon dioxide evolution was decreased. Although glucose was completely utilized in experiments with initial ferricyanide concentrations of 72 and 140 mM, in the latter case the concentration of acetate at the end of the experiment was about six times that at the former concentration of ferricyanide. Thus a high initial ferricyanide concentration did not seem to affect the fermentative path of glucose oxidation, except that the rate of ferricyanide reduction was considerably decreased, but it did interfere with the further oxidation of acetate which therefore accumulated.

The rate and total amount of ferricyanide reduced from a given amount of glucose were not affected by the presence of ferrocyanide in initial concentrations up to half the ferricyanide concentration. These results and the fact that bacterial suspensions which had been in contact with ferricyanide for several hours could still oxidize glucose readily, indicated that these complex ions were not poisonous to the bacteria.

Effect of ferricyanide on the biosynthesis of formate hydrogenlyase

When washed suspensions of *Escherichia coli* which had been grown aerobically were incubated anaerobically in the presence of tryptone or glucose then, within 1 or 3 hr, respectively, the synthesis of formate hydrogenlyase could be detected by the evolution of hydrogen. When ferricyanide was added to a suspension of bacteria in which the enzyme had already been induced, evolution of hydrogen continued, indicating that ferricyanide did not inhibit the action of formate hydrogenlyase. However, when bacteria in which the enzyme had not been induced were incubated anaerobically with either tryptone or glucose in the presence of ferricyanide (10–300 μmoles/ml.) no formate hydrogenlyase was induced.

Washed bacterial suspensions and cell-free extracts of organisms grown under a variety of growth conditions, rapidly reduced ferricyanide and evolved carbon dioxide when formate or glucose were supplied, indicating the presence of a very active formic dehydrogenase.

*Anaerobic oxidation of various substrates by washed suspension of
Escherichia coli in the presence of ferricyanide*

The results of a typical experiment where the reduction of ferricyanide by washed suspensions of *Escherichia coli* in the presence of various substrates was followed potentiometrically, are shown in Fig. 2. The bacteria used in all these experiments had been grown with succinate as energy source because glucose-grown bacteria did
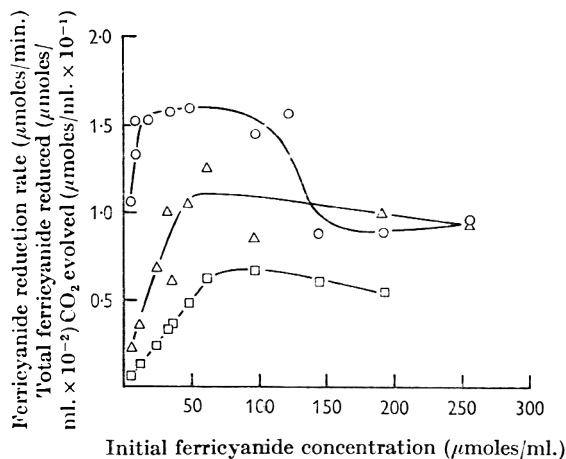
Fig. 1   Fig. 2

Fig. 1. Effect of ferricyanide concentration on glucose oxidation by washed suspension of *Escherichia coli* under anaerobic conditions. Ferricyanide reduction was measured potentiometrically. Each reaction vessel contained (per ml.): equiv. 1·28 mg dry wt. bacteria; 2 μmoles glucose; ferricyanide; phosphate buffer (pH 7·0). Incubation at 37°. CO_2 evolution measured manometrically. Values have not been corrected for endogenous respiration, which accounted for the reduction of 10–25 μmoles of ferricyanide/ml. and the evolution of 0·5–1·5 μmoles CO_2/ml., depending on the original ferricyanide concentration. Rate of ferricyanide reduction (O); total ferricyanide reduced (□); carbon dioxide evolved (△).
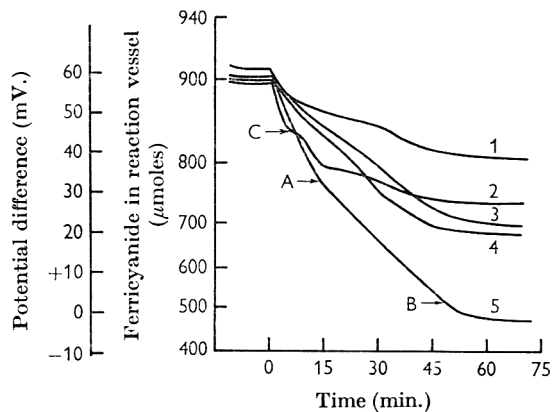
Fig. 2. Ferricyanide reduction with various substrates by washed suspensions of *Escherichia coli* under anaerobic conditions. Each reaction vessel contained: 900 μmoles ferricyanide; 100 μmoles ferrocyanide; 20 μmoles substrate; phosphate buffer (pH 7·0) to final volume 10·5 ml. At time 0, 2 ml. bacterial suspension (equiv. 16 mg dry wt. bacteria) was added. Incubation at 37°. The reduction of ferricyanide was followed potentiometrically, with the following substrates: 1, endogenous; 2, formate (a further 20 μmoles added at C); 3, acetate; 4, succinate; 5, glucose. The figure shows the actual potentiometer recordings (in millivolts) against a reference electrode immersed in half-reduced ferricyanide. The corresponding total amounts of ferricyanide in the reaction vessel have been calculated from the potential difference.

not oxidize any of the intermediates of the TCA cycle when these compounds were supplied in the reaction vessel. At point A on curve 5 (Fig. 2), where the rate of ferricyanide reduction changed sharply, the acetate produced from the glucose reached a maximum (16·5 μmoles total) and only traces of glucose remained. The acetate then gradually disappeared (2·8 μmoles at point B). In Table 1 the amounts of ferricyanide reduced and of carbon dioxide evolved from the oxidation of 1 mole substrate are given. In most cases the substrates were almost completely oxidized. Acetate accumulated during the oxidation of glucose but had disappeared by the

end of the experiment. However, several substrates, particularly α-oxoglutarate, pyruvate and oxaloacetate were only partially used by the end of the experiment. This was probably caused by the relative impermeability of the bacteria to these substrates under anaerobic conditions with ferricyanide as the hydrogen acceptor. For pyruvate and α-oxoglutarate only 5 μmoles of a total 20 μmoles were used by the end of the experiment. During the oxidation of the other substrates neither α-oxoglutarate nor pyruvate accumulated in the medium, indicating that these compounds were metabolized rapidly inside the organisms. When pyruvate, succinate, lactate or oxaloacetate were used a small amount of acetate was detected in the medium at the end of the experiment. In most cases the carbon dioxide evolved/ mole substrate was higher than expected from the corresponding amounts of ferricyanide reduced. Under these conditions it is possible that some non-oxidative decarboxylation occurred. With glucose and acetate as substrates the carbon dioxide evolved was greater than the theoretical amount for complete oxidation. This may have been the result of increased oxidation of endogenous metabolites during the oxidation of the glucose or acetate (Stoppani & Ramos, 1964).

Table 1. *Ferricyanide reduction and carbon dioxide evolution during the oxidation of various substrates by washed suspensions of Escherichia coli under anaerobic conditions*

Ferricyanide reduction was measured potentiometrically in a system: equiv. 16 mg. dry wt. bacteria; 20 μmoles substrate; 900 μmoles ferricyanide, 100 μmoles ferrocyanide; phosphate buffer (pH 7·0) to a final volume 12·5 ml.

The CO_2 evolution was measured manometrically using the same concentrations of reactants except that the substrate concentration was doubled.

All results are corrected for the endogenous respiration and are the mean of at least five experiments.

Substrate	H equiv. for complete oxidation/ mole	Moles ferricyanide reduced/mole substrate	Moles CO_2 evolved/ mole substrate
6 carbon			
glucose	24	20	6·5
5 carbon			
α-oxoglutarate	16	7	4
4 carbon			
succinate	14	5·7	3·5
fumarate	12	10	3·56
malate	12	7	3·3
oxaloacetate	10	1·25	1·06
3 carbon			
lactate	12	6	1·4
pyruvate	10	11	3
2 carbon			
acetate	8	8·2	2·3
1 carbon			
formate	2	1·9	0·9

The total oxygen uptake and carbon dioxide output with various substrates in the presence or absence of ferricyanide is shown in Table 2. The presence of ferricyanide depressed slightly the oxygen uptake and increased the carbon dioxide

evolution, showing that the amount of substrate assimilated was decreased. Under anaerobic conditions, however, with ferricyanide as hydrogen acceptor, the carbon dioxide evolved/mole substrate indicated that no assimilation of substrate occurred (Table 1). The decreased oxygen uptake in the presence of ferricyanide was probably due to ferricyanide competition with oxygen as a hydrogen acceptor. On this basis and from the amount of ferricyanide reduced (7 μmoles/μmole glucose) about one-third of the glucose was oxidized with ferricyanide as hydrogen acceptor, even though oxygen was present.

Table 2. *Effect of ferricyanide on the oxygen uptake and carbon dioxide evolution in the presence of various substrates by washed suspensions of Escherichia coli. Experimental details as for Table 1*

Substrates	Ferri-cyanide added	Moles oxygen uptake/mole substrate	Moles CO_2 evolved/mole substrate
Glucose	—	3·2	3·2
	+	2·0	3·4
Succinate	—	1·21	1·1
	+	1·30	2·1
Pyruvate	—	0·60	0·67
	+	0·55	1·0
Acetate	—	1·42	1·13
	+	1·16	1·92

Effect of ferricyanide on energy production

In some experiments with washed suspensions of *Escherichia coli* it was noticed that at high concentrations of glucose, less ferricyanide was reduced/mole glucose than at low glucose concentrations. Under these conditions acetate accumulated, thereby accounting for the smaller amount of ferricyanide reduced. However, it was not clear why acetate should have accumulated. The same effect was observed when acetate was used instead of glucose. When either a high initial concentration of acetate was used or small amounts were added at intervals it was found that a maximum limit of about 60 μmoles acetate was oxidized by the bacterial suspension with ferricyanide as hydrogen acceptor. The addition of glucose to the system resulted in further ferricyanide reduction, but the rate and total amount was very low. Further experiments showed that the amount of acetate which was oxidized in the presence of ferricyanide was related to the dry weight bacteria in the reaction vessel.

To determine whether or not the reduction of ferricyanide by *Escherichia coli* was coupled to ATP formation, experiments were made with actively growing bacteria. In the absence of oxygen, ferricyanide did not support the growth of *E. coli* with succinate as energy source in an otherwise complete medium. The effect of ferri-cyanide on the anaerobic growth yield and the fermentation products of *E. coli* with glucose as energy source is shown in Table 3. In the presence of ferrocyanide (40 μmoles/ml.) the growth yield was 44 g. dry wt. bacteria/mole glucose. At about pH 7 the main fermentation products of *E. coli* are acetate, ethanol, formate and lactate (Mickelson & Werkman, 1938; Wood, 1961). In this type of fermentation pyruvate is split by a thioclastic reaction to acetyl-CoA and formate. Half of the

acetyl-CoA formed is reduced to ethanol (Dawes & Foster, 1956) and the remainder is converted to acetate with the production of ATP. In the presence of hydrogen acceptors ethanol is not produced and another gain in ATP occurs. Our results indicate that ferricyanide did act as a hydrogen acceptor under these conditions. This was also supported by the observation that acetate accumulated in the medium and only small amounts of reduced products such as ethanol were found. A decrease in the amount of ferricyanide present resulted in a decrease in the amount of acetate formed and an increase in the amount of ethanol. These values (Table 3) were determined in the supernatant fluid when the bacterial concentration had reached its maximal value. No hydrogen was evolved either during or after growth when ferricyanide was present. This result with growing bacteria confirmed the observation with washed suspensions that ferricyanide prevented the formation of formate hydrogenlyase. In experiments where ferricyanide was not present the cessation of growth was followed by the rapid evolution of hydrogen (0·96 moles/mole glucose) and of carbon dioxide which reached a value of 1·3 moles/mole glucose.

Table 3. *Anaerobic growth yields and end products of glucose metabolism by Escherichia coli growing in the presence and absence of ferricyanide*

The tryptone liver-digest medium contained glucose (5 μmoles/ml.) and ferricyanide (0, 10 or 40 μmoles/ml.) Incubation at 37°. All values per mole of glucose.

	Ferricyanide (moles/mole of glucose)		
	0	2	8
Growth yield (g. dry wt. bacteria:	38	33	23
Acetate (moles):	1·0	1·2	1·9
Ethanol (moles):	1·0	0·8	0·3
Formate (moles):	1·7	1·6	0·0
Pyruvate (moles):	0·06	*	0·2
CO_2 evolved (moles):	0·4	*	1·5
Ferricyanide reduced (moles):	0·0	2·0	5·0

* Values not determined.

Under aerobic conditions the presence of ferricyanide decreased the growth rate on glucose and on succinate, and the maximum growth yield, which was only about one-quarter of the value obtained in the absence of ferricyanide, with both substrates. Ferrocyanide decreased the growth rate but did not affect the anerobic growth yield and had only a slight effect on the growth yield when the bacteria were grown aerobically on glucose or succinate.

DISCUSSION

From the results of this work it is clear that ferricyanide can act as hydrogen acceptor with washed suspensions or growing cultures of *Escherichia coli*. The results with washed suspensions of bacteria show that the oxidation of glucose was almost complete in the presence of ferricyanide and absence of oxygen, indicating that the TCA cycle was functioning satisfactorily under these conditions. This is confirmed by the fact that most of the intermediates of the TCA cycle were metabolized under these conditions, although in the case of α-oxoglutarate and pyruvate their use

appeared to be restricted. This may have been due to a shortage of energy, caused by the presence of the ferricyanide, needed either for the synthesis of a permease system or for the action of this 'active transport'. Systems for active transport of several carbohydrates and amino acids have been reported for *E. coli* (Kepes & Cohen, 1962).

Our results, especially those from the growth experiments, show that ferricyanide reduction was not coupled to ATP formation. The growth yield under anaerobic conditions was decreased in the presence of ferricyanide, indicating that at least some of the energy produced by substrate-level phosphorylation was being dissipated. A similar phenomenon was observed when nitrite was used as a hydrogen acceptor by *Aerobacter aerogenes* (Hadjipetrou, 1965). In the presence of these hydrogen acceptors perhaps a proteus-type cleavage of pyruvate takes place (Gunsalus, 1954; Moyed & O'Kane, 1956) where free acetate is produced without the formation of an intermediate high energy moiety. Our results confirm the conclusion of Hager (1957) for the oxidation of pyruvate by extracts of *Escherichia coli* and ferricyanide as the hydrogen acceptor. However, even if formate is produced it is rapidly decomposed by formate dehydrogenase, which is very active in the presence of ferricyanide, and cannot be detected in the medium (Table 3).

When insufficient ferricyanide is present then, when it is exhausted, formate accumulates because formate hydrogenlyase synthesis is repressed under these conditions. A similar repression of the synthesis of this enzyme has been observed with all other hydrogen acceptors which have been examined, i.e. oxygen, nitrate, nitrite (Gest, 1954; Pichinoty, 1962; Billen, 1951; Pinsky & Stokes, 1952; Hadjipetrou, unpublished results).

Since ferricyanide reduction is not coupled with ATP production the amount of glucose or other substrates which can be completely oxidized via the TCA cycle is limited by the amount of ATP or other energy-yielding compounds present in the bacteria at the start of the experiments. Further evidence for the uncoupling effect of ferricyanide comes from the results for the oxidation of acetate which proceeds as long as glucose or other endogenous material is present and is being oxidized at the same time. Although acetate can readily enter the organism, energy is required for its conversion into acetyl-CoA, which can then enter the TCA cycle. The same effect has been observed for the oxidation of acetate by baker's yeast in the presence of dinitrophenol (Stoppani & Ramos, 1964). At the concentrations used, ferricyanide and its reduced product ferrocyanide do not appear to inhibit directly any of the enzymes of the glycolytic pathway and TCA cycle. It is not yet clear whether the enzyme responsible for ferricyanide reduction is similar to the reductases coupling with quinones and triphenyltetrazolium chloride which have already been isolated from *Escherichia coli* and shown to be flavoproteins (Brodie & Gots, 1952; Wosilait & Nason, 1954; Kashket & Brodie, 1963).

REFERENCES

BILLEN, D. (1951). The inhibition by nitrate of enzyme formation during growth of *E. coli*. *J. Bact.* **62**, 793.

BRODIE, A. F. & GOTS, J. S. (1952). Reduction of tetrazolium salts by an isolated bacterial flavoprotein. *Science* **116**, 588.

DAWES, E. A. & FOSTER, S. M. (1956). The formation of ethanol in *E. coli*. *Biochim. biophys. Acta* **22**, 253.

DOLIN, M. I. (1961). Cytochrome-independent electron transport enzymes of bacteria. In *The Bacteria*, Vol. 2, p. 425. New York and London: Academic Press.

EGAMI, F., ISHIMOTO, M. & TANIGUCHI, S. (1961). The electron transfer from cytochromes to terminal electron acceptor in nitrate respiration and sulphate respiration. In *Haematin Enzymes*. Vol. 2, p. 392. London: Pergamon.

FRIEDEMANN, T. E. & HAUGEN, G. (1943). The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.

GEST, H. (1954). Oxidation and evolution of molecular hydrogen by micro-organisms. *Bact. Rev.* **18**, 43.

GRAY-YOUNG, T., HADJIPETROU, L. P. & LILLY, M. D. (1966). The theoretical aspects of biochemical fuel cells. *Biotech. Bioeng.* (in Press).

GUNSALUS, I. C. (1954). Group transfer and acyl-generating functions of lipoic acid derivatives. In *The Mechanism of Enzyme Action*. Ed. by W. D. McGilroy & B. Glass, p. 545. Baltimore Maryland, U.S.A.: Johns Hopkins Press.

HADJIPETROU, L. P. (1965). *Relation between energy production and aerobic growth of bacteria*. Ph.D. thesis University of Utrecht, Netherlands.

HADJIPETROU, L. P. & STOUTHAMER, A. H. (1965). Energy production during nitrate respiration by *Aerobacter aerogenes*. *J. gen. Microbiol.* **38**, 29.

HAGER, L. P. (1957). Trypsin activation of a ferricyanide linked pyruvic acid oxidation. *J. biol. Chem.* **229**, 251.

KASHKET, E. R. & BRODIE, A. F. (1963). Oxidative phosphorylation in fractionated bacterial systems. X Different roles for the natural quinones of *E. coli* w in oxidative metabolism. *J. biol. Chem.* **238**, 2564.

KEPES, A. & COHEN, G. N. (1962). Permeation. In *The Bacteria*, Vol. 4, p. 179. New York and London: Academic Press.

LINNANE, A. W. & WRIGLEY, C. W. (1963). Fragmentation of the electron transport chain of *E. coli*. Preparation of a soluble formate dehydrogenase-cytochrome b_1 complex. *Biochim. biophys. Acta* **77**, 408.

MARK, V. (1959). Enzymatic method for determination of dextrose. *Clin. chim. Acta* **4**, 395.

MICKELSON, M. & WERKMAN, C. H. (1938). Influence of pH on the dissimilation of glucose by *Aerobacter indologenes*. *J. Bact.* **36**, 67.

MOYED, H. S. & O'KANE, D. J. (1956). Enzymes and coenzymes of the pyruvate oxidase of Proteus. *J. biol. Chem.* **218**, 831.

NASON, A. (1962). Symposium on metabolism of inorganic compounds. II. Enzymatic pathways of nitrate, nitrite and hydroxylamine metabolisms. *Bact. Rev.* **26**, 16.

NEISH, A. C. (1952). Analytical methods for bacterial fermentations. Saskatoon: National Research Council of Canada.

PICHINOTY, F. (1962). Inhibition par l'oxygène de la biosynthèse et de l'activité de l'hydrogénase et de l'hydrogénlyase chez les bactéries anaèrobies facultatives. *Biochim. biophys. Acta* **64**, 111.

PINSKY, M. J. & STOKES, J. L. (1952). Requirements for formic hydrogenlyase adaptation in nonproliferating suspensions of *E. coli*. *J. Bact.* **64**, 151.

POSTGATE, J. R. (1965). Recent advances in the study of the sulphate-reducing bacteria. *Bact. Rev.* **29**, 425.

QUASTEL, J. H., STEPHENSON, M. & WHETHAM, M. D. (1925). Some reactions of resting bacteria in relation to anaerobic growth. *Biochem. J.* **19**, 304.

SENEZ, J. C. (1962). Some considerations on the energetics of bacterial growth. *Bact. Rev.* **26,** 95.

STOPPANI, A. O. M. & RAMOS, E. H. (1964). The action of 2,4-dinitrophenol on the metabolism of actate and pyruvate in baker's yeast. *Arch. Biochem. Biophys.* **105,** 470.

WOOD, W. A. (1961). Fermentation of carbohydrates and related compounds. In *The Bacteria*, Vol. 2, p. 59. New York and London: Academic Press.

WOSILAIT, W. D. & NASON, A. (1954). Pyridine nucleotide-menadione reductase from *E. coli. J. biol. Chem.* **208,** 785.

Thymine Starvation and Genetic Damage in *Escherichia coli*

By M. G. R. HART

Department of Botany, The Queen's University, Belfast, Northern Ireland

SUMMARY

Two *thy*⁻ Hfr strains of *Escherichia coli* K12 were mated with stationary phase w 677 F⁻ at intervals during thymine starvation. With both strains, Hfr bacteria retained their capability of chromosome transfer for a period after loss of colony-forming ability. Eventual loss of transfer capability in strain HfrC *thy*⁻ was associated with inactivation of some early stage of the transfer process. Thymine starved HfrC *thy*⁻ transferred the genetic markers *pro, thr, leu* and *thi* with a normal gradient but HfrB1 *thy*⁻ showed marker inactivation. The extent of marker inactivation in this strain was consistent with the presence of 10–20 breaks/chromosome. It is suggested that the strains may have behaved differently in these experiments as a result of differences in their ability to modify primary genetic lesions resulting from thymine starvation.

INTRODUCTION

Evidence has accumulated which suggests that 'thymineless death' (Cohen & Barner, 1954) may be associated with nuclear damage. Maaløe (1961) showed that the susceptibility of a bacterium to thymineless death was dependent on its ability to synthesize DNA at the time thymine was withdrawn. Fuerst & Stent (1956) showed that the lethal effects of thymine starvation and the decay of ³²P in the bacterial nucleus were additive. Others (Gallant & Suskind, 1961; Rassmussen & Painter, 1963) showed a similar connection between the effects of thymine starvation and ultraviolet (u.v.) irradiation. Stacey, Symonds & Atkinson (1966) reported a number of other similarities between u.v. death and thymineless death. Several groups have reported a mutagenic effect of thymine starvation (Coughlin & Adelberg, 1956; Weinberg & Latham, 1956; Kanazir 1958) though this has not been confirmed in the present author's laboratory using thymine⁻ strains of *Escherichia coli* K12, all of which died rapidly under conditions of thymine deprivation.

Attempts to obtain specific evidence about possible nuclear damage have been rather unsuccessful. Luzatti & Revel (1962) detected no physical differences between normal and 'thymineless' DNA. Freifelder & Maaløe (1964), who used a technique which had been successful in detecting single-strand breaks in T bacteriophage DNA (Davison, Freifelder & Holloway, 1964) obtained no evidence for such breaks in the DNA extracted from thymine-starved bacteria. However, DNA extracted from thymine-starved *Bacillus subtilis* showed a decreased efficiency of transformation (Sicard & Anagnostopoulos, 1964). Meningmann & Sybalski (1962) also obtained this effect with DNA extracted from *B. subtilis* treated with 5-fluorodeoxyuridine as an inhibitor of thymidylate synthetase; the presence of single-strand breaks was inferred from sedimentation data and viscosity measurements. However, in such

experiments it is difficult to rule out the possible effects on the bacterial DNA of normal or defective prophages which are induced as a consequence of thymine deprivation (Korn & Weissbach, 1962; Melechen & Skaar, 1962; Sicard & Devoret, 1962).

Jacob & Wollman (1958), in their experiments on the conjugation process, were able to show that ^{32}P decay within the Hfr nucleus and u.v. irradiation of the Hfr bacterium before mating had distinctive effects on the genetic continuity of the chromosome. Intranuclear ^{32}P decay caused inactivation of the various genetic markers, increasing the steepness of the normal gradient of transfer. U.v. irradiation of the male cell did not affect the transfer process, but decreased the extent to which portions of chromosome carrying more than one marker were integrated into recombinant genotypes.

Because of the related effects of thymine starvation, intranuclear ^{32}P decay and u.v. irradiation, it might be expected that they would influence transfer and recombination in a similar way. The experiments to be described were undertaken to investigate this possibility.

METHODS

Organisms. The following strains of *Escherichia coli* were used; K12 HfrC *thy⁻ met⁻* (λ)$^+$ λ-*sens str-s*, transferring chromosomal markers in the order-origin, *pro* (10 min.) *leu, thr, thi*; K12 HfrB1 (Broda) *thy⁻ met⁻* (λ)$^-$ λ-*res str-s* transferring chromosomal markers in the order-origin, *pro* (5 min.), *leu, thr, thi*; 15T⁻ *thy⁻* (Cohen & Barner, 1954); K12 W677 *pro⁻ thr⁻ leu⁻ thi⁻ str-r* F⁻. The *thy⁻* strains of HfrC and HfrB1 were obtained using aminopterin (Okada, Yanagisawa & Ryan, 1961) and both had a high requirement for thymine (50 μg./ml.). Abbreviations: *leu⁻*— leucineless, *met⁻*—methionineless, *pro⁻*—prolineless, *sens*—sensitive, *str-s*—streptomycin sensitive, *str-r*—streptomycin resistant, *thi⁻*—thiamineless, *thr⁻*—threonineless, *thy⁻*—thymineless.

Culture media. Bacteria were grown in Oxoid Nutrient Broth No. 2 or in minimal M9 medium containing (g./l.) Na₂HPO₄, 6; KH₂PO₄, 3; NaCl, 0·5; NH₄Cl, 1; MgSO₄, 0·2; glucose, 4; dissolved in 1 l. distilled water. Amino acid supplements were added to M9 medium at a final concentration of 20 μg./ml. and thiamin at 0·2 μg./ml. Thymine was used in nutrient and minimal media at 80 μg./ml.

'Thymineless broth' was nutrient broth in which *Escherichia coli* 15T⁻ had been grown until extensive filamentation was observed. Two periods of growth, each followed by centrifugation and re-autoclaving were necessary to obtain deficiency symptoms indicative of a low thymine content of the broth.

Oxoid Blood Agar Base No. 2 with added thymine was used for colony counts of viable bacteria.

Recombinants were assayed by plating on G and T agar (Okada *et al.* 1961) with appropriate nutrient supplements, nutrient broth 0·5 %, v/v (Gross, 1963) and streptomycin 250 μg./ml.

Growth of cultures. Cultures of all strains were prepared by inoculation of part of a single colony from a recently prepared streak plate into a complete liquid medium and gently agitated during overnight incubation. Log phase cultures were prepared from overnight cultures by diluting 1/10 into similar medium prewarmed to 37° and incubating with aeration for 90 min.

Thymine starvation. Transfer of bacteria to thymine-deficient medium was made by using membrane filtration. Bacteria were washed three times in thymine-deficient medium before re-suspension. All operations were done at 37°; all media and equipment were pre-warmed to this temperature before use.

Mating procedure. A sample (0·2 ml.) of Hfr suspension was added to a mixture of 1 ml. overnight culture of *Escherichia coli* w 677 in unsupplemented broth and 1 ml. fresh broth supplemented with 160 μg./ml. thymine. The mixture was rotated gently on an inclined turntable for 1 hr. Samples were then removed for plating.

Pairing experiments. Experiments on pair formation were made by the technique of Jacob & Wollman (1961).

Pulse mating was done by the method of de Haan & Gross (1962).

RESULTS

Effects of thymine starvation on recombinant formation by HfrC thy⁻

Figure 1 shows the results of mating HfrC *thy⁻* bacteria at intervals during thymine starvation, together with the viability loss which occurred. No inactivation of the markers *thr leu* was obtained relative to the marker *pro*. Isolation and testing of a number of *pro⁺* colonies gave no indication of a decreased frequency of transfer of the *thi* character by thymine-starved bacteria. Other experiments showed that the rate of decline of *pro⁺ thi⁺* recombinants was the same as that of *pro⁺*. In this strain, the kinetics of transfer and recombination were apparently unaffected by thymine starvation.

Viability of the HfrC population declined more readily than the number of recombinants which it produced and after about 3 hr, the number of recombinants/ml. of the mating mixture was almost the same as the number of viable HfrC bacteria. This could have resulted from a greatly increased efficiency of recombinant formation by the surviving bacteria, from a selection against a non-fertile fraction of the Hfr population by the lethal action of thymine starvation, or from a participation in the recombination process by 'dead' bacteria unable to form colonies. Recombination by 'dead' bacteria is most likely as in later experiments (Fig. 5b) with HfrB1 *thy⁻* (λ)⁻ the number of recombinants formed by thymine-starved populations greatly exceeded the number of viable male bacteria present. The method therefore allows an examination of the kinetics of transfer by 'dead' bacteria and should be capable of detecting genetic damage accompanying thymineless death. Plating on minimal medium or incubation at 42° or 25° did not give significantly different estimates of viability. With strain HfrC *thy⁻*, the discrepancy between the rates of viability loss and fertility loss was not so great when minimal medium was used and experiments were therefore always made with broth media.

The discrepancy in rates did not persist. When thymine-starved bacteria were incubated for 30 min. in thymine-supplemented broth before mating the results shown in Fig. 2 were obtained. No marker inactivation occurred but the rates of loss of viability and fertility were similar. The 30 min. treatment in complete medium had resulted in loss of fertility by the dead bacteria. This was confirmed in an experiment in which bacteria starved of thymine for 180 min. were incubated in complete medium for various periods before mating. The results are shown in Fig. 3. A decrease in fertility of the thymine-starved bacteria occurred for a

15–20 min. period, after which no decline was observed. A small increase in viable count (less than one doubling) occurred during the 30 min. period of the experiment. The rate of loss of fertility in this experiment was higher than that which normally occurred during thymine starvation (see Figs. 1, 2). Possibly DNA synthesis accelerated the decline.

Fig. 1   Fig. 2
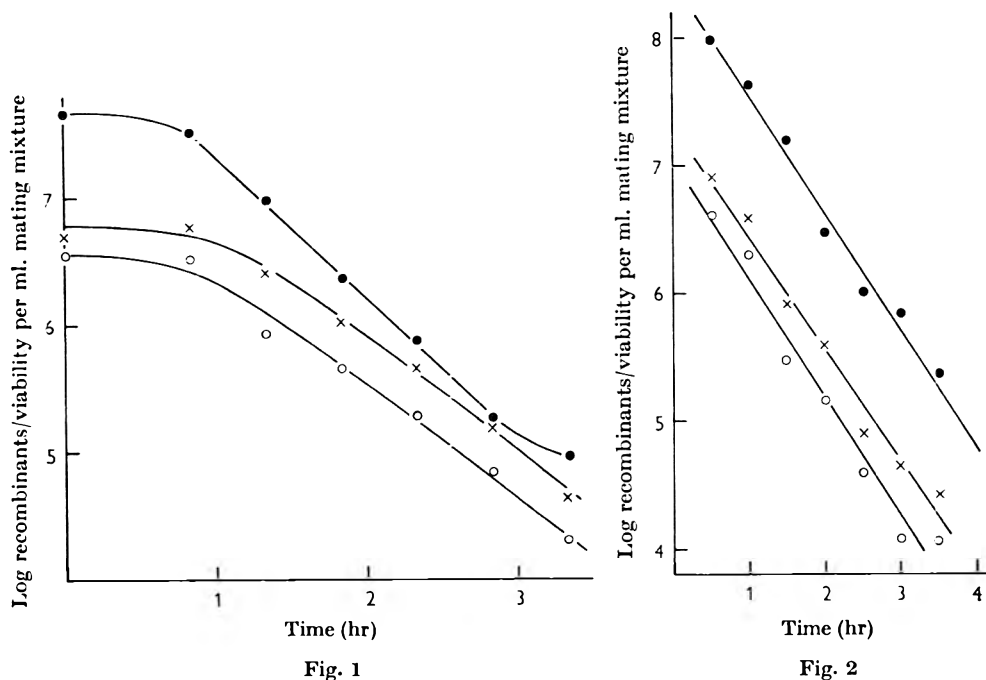
Fig. 1. Viability loss and recombinant formation by thymine-starved HfrC *thy⁻*. A 10 ml. log phase culture of HfrC *thy⁻* in thymine-supplemented broth was washed and re-suspended in the same volume of thymineless broth. Samples were taken at intervals during thymine starvation for (*a*) colony counts, (*b*) 60 min. mating with w677 and subsequent plating for *pro⁺* and *thr⁺ leu⁺* recombinants. Log viable count ●—●, log. *pro⁺* ×—× and log *thr⁺ leu⁺* ○—○ are given per ml. of the mating mixture which contained: 0·2 ml. HfrC in thymineless broth, 1 ml. overnight culture of w677 in unsupplemented broth and 1 ml. fresh broth supplemented with 160 µg./ml. thymine.

Fig. 2. Effect of pre-incubation in complete medium before mating on recombinant formation by thymine-starved HfrC *thy⁻* bacteria. Experimental procedure and symbols as in Fig. 1. except that samples of the HfrC suspension were held at 37° with added thymine (80 µg./ml.) for 30 min. before the addition of the F⁻ bacteria.

The stage of the conjugation process affected by thymine starvation

The results suggest that thymineless death occurred in at least two recognizable stages; a first stage in which the bacterium lost ability to form colonies (it has so far not been possible to devise conditions which will reverse this step in this strain; M. H. L. Green, personal communication), and a second stage in which the bacterium loses ability to form recombinants. Possible ways in which the bacterium might be damaged in the second stage are as follows: (*a*) those which interrupt the transfer process and result in apparent marker inactivation, e.g. chromosome breakage, pair instability resulting in premature separation, metabolic failure, lysis; (*b*) those

which prevent initiation of transfer but do not affect the process of transfer itself, e.g. inability to form effective pairs as a result of nuclear damage of a kind which would not cause marker inactivation. The fact that marker inactivation did not occur makes (a) damage less likely and suggests that the second stage during which fertility was lost might result from specific damage to the pairing or transfer mechanism.

Figure 4*a* shows the result of a pulse-mating experiment with HfrC *thy⁻* bacteria starved of thymine for 180 min. Except for a small increase in the time of entry of the proline marker, which might have been due to a thymine requirement in transfer
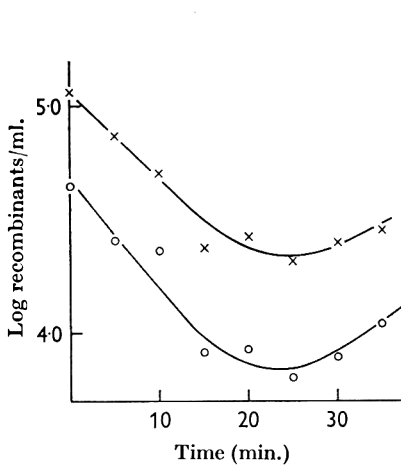
Fig. 3
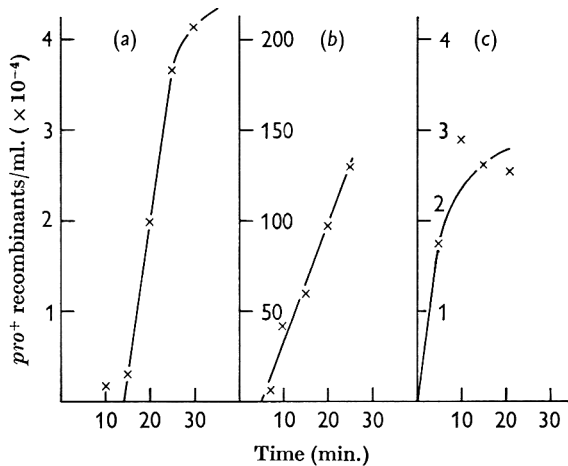
Fig. 4

Fig. 3. Effects of periods of incubation in broth + thymine on recombinant formation by thymine starved HfrC *thy⁻* bacteria. Symbols as in Fig. 1. HfrC *thy⁻* bacteria starved of thymine for 3 hr in *thy⁻* broth, then 80 μg./ml. thymine added and cultures held at 37° for various intervals before mating with w677 as in Fig. 1 procedure.

Fig. 4. (a) Pulse mating experiment showing increase of *pro⁺* recombinants after mating HfrC *thy⁻* starved of thymine 3 hr. (b) Pair formation by normal bacteria of HfrC *thy⁻*. (c) Pair formation by HfrC *thy⁻* after 3 hr thymine starvation.

initiation (Pritchard, 1965) the kinetics of transfer are normal for this type of experiment. The 15 min. spread of transfer times found by de Haan & Gross (1962) occurred and there was no suggestion of a decrease in the rate at which new transfers were initiated during this 15-min. period. Damage of type (a) excluding actual chromosome breakage might be expected to cause a decrease in the rate of initiation of new transfers. Such experiments lend support to the idea that genetic transfer by 'thymineless dead' bacteria is not inactivated once effective pairs have been formed.

The results of pairing experiments also suggest that the decline in fertility which occurred in HfrC *thy⁻* after thymineless death was the result of loss of ability to form effective pairs. Figures 4*b* and 4*c* show the results of pairing experiments with this strain after 0 and 180 min. of thymine starvation. The ability of the thymine-starved population to form effective pairs was greatly decreased with time, whilst the unstarved population retained its initial pair forming ability.

Effects of thymine starvation on recombinant formation
by strain HfrB1 thy⁻

A second Hfr strain, B1 *thy⁻*, which was $(\lambda)^-\,\lambda^-$ res˙and therefore not susceptible to induction of phage λ during thymine starvation was chosen for comparison. This strain did not lyse when thymine was returned after a period in thymineless broth, but its behaviour in minimal medium indicated that the presence of defective prophages was not entirely eliminated. HfrB1 transfers almost the same piece of chromosome as HfrC, and with the same orientation.
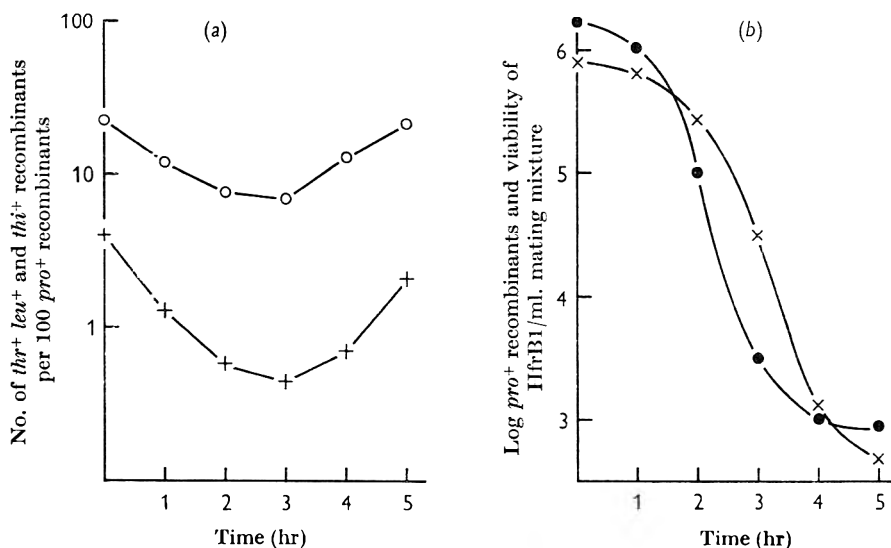
Fig. 5. (*a*) Effect of thymine starvation of HfrB *thy⁻* on the proportion of distal markers inherited in crosses with w 677. (*b*) Viability loss and recombinant formation by thymine starved HfrB1 *thy⁻*. Method for both Fig. 5(*a*) and (*b*) was as described for Fig. 1 except that the Hfr strain was grown overnight in minimal medium, brought to log phase in 10 ml. of the same medium and the whole volume re-suspended in 50 ml. minimal medium minus thymine after filtration and washing.

Marker inactivation was obtained with this strain. Figure 5*a* shows the decline in the percentage of the distal markers inherited, relative to the number of *pro⁺* recombinants. No decrease in the proportion of *pro⁺ thi⁺* recombinants relative to the number of *thi⁺* occurred. The degree of marker inactivation increased to a maximum at 130 min, after which it declined. At 300 min. the figures are close to those obtained using unstarved bacteria. This indicates that transfer by the immune fraction of the population was normal and that marker inactivation was restricted to that fraction of bacteria which were incapable of colony formation. The time of maximum marker inactivation coincided with the time at which most dead bacteria were still capable of transfer (Fig. 5*b*).

If the 'immune' bacteria are neglected, and it is assumed that the amount of marker inactivation observed is entirely due to random chromosome breaks, it can be calculated that between 10 and 20 breaks/transferred chromosome would be present. This estimate would be well below the limit of detection quoted by Freifelder & Maaløe (1964).

One cannot be entirely certain that marker inactivation is not the result of some factor other than chromosome breakage. Decreased stability of mating pairs, stoppage of transfer or decreased rate of transfer would all give similar results. However, it is difficult to reconcile the absence of marker inactivation in strain HfrC *thy*⁻ with any of these explanations. In addition, pulse-mating experiments with strain HfrB1 *thy*⁻ gave identical results whether with starved or unstarved bacteria. No decline in the rate of increase of recombinants during the 15 min. spread was detected and thymine starvation had no effect on the time of entry of successive markers indicating that the rate of transfer was unchanged in thymine-starved populations.

DISCUSSION

With both Hfr strains of *Escherichia coli* there was a clear separation between the stage at which a bacterium was no longer able to form colonies and its loss of fertility. The absence of marker inactivation in HfrC suggests that fertility was eliminated by damage specifically affecting transfer initiation or functions associated with it. Nuclear damage may be involved, though the results give no indication as to whether such damage would be the primary cause of thymineless death.

No evidence was obtained in either strain for a stimulation of recombination such as has been demonstrated by Jacob & Wollmann (1958) with u.v. irradiation of donor strains or ³²P decay in zygotes. This is surprising since Gallant & Spottswood (1965) showed strong recombinogenic effect of thymine starvation in *Escherichia coli* merodiploids. The results of the present experiments suggest that if genetic damage is transferred in the donor fragment it has no effect on the activity of the recombinase systems of the recipient. The final outcome may depend on some characteristic of the recipient bacterium used.

If the marker inactivation observed in strain HfrB1 *thy*⁻ is entirely a consequence of chromosome breaks, the results are not compatible with the suggestion of Maaløe (1961) that thymine starvation might cause damage at the replicating point. This hypothesis would predict a single break/chromosome in bacteria having a single replicating point, and the amount of inactivation observed could only be accounted for on a single-hit hypothesis if the damage was very extensive. A number of individual lesions, possibly occurring during transcription as suggested by Galland & Suskind (1962), would seem to be more likely.

A possible explanation for the difference observed between the two strains may lie in a differing ability to modify primary genetic lesions. Single strand breaks blocking transfer might occur as a result of the activity of an enzyme such as that which has been shown to excise pyrimidine dimers in u.v. irradiated *Escherichia coli* (Setlow & Carrier, 1964). Thymine deprivation might prevent completion of the repair by preventing DNA synthesis on the remaining single strand template; a single strand gap which prevented chromosome transfer would thus be produced. Preliminary experiments with HfrB1 *thy*⁻ have shown that marker inactivation can be produced by u.v. irradiation followed by a short period of thymine starvation. Experiments now in progress suggest that the strains used in the present experiments and their parent *thy*⁺ strains do differ in their ability to repair u.v. induced lesions.

REFERENCES

COHEN, S. S. & BARNER, H. D. (1954). Studies on unbalanced growth in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **40**, 885.

COUGHLIN, C. A. & ADELBERG, E. A. (1956). Bacterial mutation induced by thymine starvation. *Nature, Lond.* **178**, 531.

DAVISON, P. F., FREIFELDER, D. & HOLLOWAY, B. W. (1964). Interruptions in the polynucleotide strands of bacteriophage DNA. *J. mol. Biol.* **8**, 1.

FREIFELDER, J. D. & MAALØE, O. (1964). Energy requirement for thymineless death in cells of *Escherichia coli*. *J. Bact.* **88**, 987.

FUERST, C. R. & STENT, G. S. (1956). Inactivation of bacteria by decay of incorporated radioactive phosphorus. *J. gen. Physiol.* **40**, 73.

GALLANT, J. & SPOTTSWOOD, T. (1965). The recombinogenic effect of thymidylate starvation in *Escherichia coli* merodiploids. *Genetics* **52**, 107.

GALLANT, J. & SUSKIND, S. R. (1961). Relationship between thymineless death and ultraviolet inactivation in *Escherichia coli*. *J. Bact.* **82**, 187.

GALLANT, J. & SUSKIND, S. R. (1962). R.N.A. synthesis and thymineless death. *Biochim. biophys. Acta* **55**, 627.

GROSS, J. D. (1963). The effect of unbalanced growth on recombinant formation in *Escherichia coli*. *Genet. Res.* **4**, 457.

DE HAAN, P. G. & GROSS, J. D. (1962). Transfer delay and chromosome withdrawal during conjugation in *Escherichia coli*. *Genet. Res.* **3**, 251.

JACOB, F. & WOLLMAN, E. L. (1958). Genetical and physical determinations of chromosomal segments in *Escherichia coli*. *Symp. Soc. exp. Biol.* **12**, 75.

JACOB, F. & WOLLMAN, E. L. (1961). *Sexuality and The Genetics of Bacteria*. p, 137. New York: Academic Press.

KANAZIR, D. (1958). Apparent mutagenicity of thymine deficiency. *Biochim. biophys. Acta* **30**, 20.

KORN, D. & WEISSBACH, A. (1962). Thymineless induction in *Escherichia coli* K12 (λ). *Biochim. biophys. Acta* **61**, 775.

LUZATTI, D. & REVEL, C. (1962). Effet léthal de la carence en thymine: état de l'acide deoxyribonucléique au cours de cette carence. *Biochim. biophys. Acta* **61**, 305.

MAALØE, O. (1961). The control of normal DNA replication in bacteria. *Cold Spring Harb. Symp. quant. Biol.* **26**, 45.

MELECHEN, N. E. & SKAAR, P. D. (1962). The provocation of an early step of induction by thymine deprivation. *Virology* **16**, 21.

MENNIGMANN, H. & SYBALSKI, W. (1962). Molecular mechanism of thymineless death. *Biochem. biophys. Res. Comm.* **9**, 398.

OKADA, T., YANAGISAWA & RYAN, F. J. (1961). A method for securing thymineless mutants of *Escherichia coli*. *Z.-Vererbungslehre* **92**, 403.

PRITCHARD, R. H. (1965). The relationship between conjugation, recombination and DNA synthesis in *Escherichia coli*. *10th Int. Congress Genetics. Genetics Today* **2**, 56.

RASMUSSEN, R. E. & PAINTER, R. B. (1963). On the early onset of thymineless death occurring after exposure to ultra-violet light. *Biochim. biophys. Acta* **76**, 157.

SETLOW, R. B. & CARRIER, W. L. (1964). The disappearance of thymine dimers from DNA: an error correcting mechanism. *Proc. natn. Acad. Sci. U.S.A.* **51**, 226.

SICARD, N. & ANAGNOSTOPOULOS, C. (1964). Activité transformante de l'acide desoxyribonucléique de *Bacillus subtilis* lors de la carence en thymine. *C. r. Acad. Sci. Paris* **259**, 4173.

SICARD, N. & DEVORET, R. (1962). Effets de la carence en thymine sur les souches d'*Escherichia Coli* lysogènes K12T⁻ et colicinogène 15T⁻. *C. r. Acad. Sci. Paris* **255**, 1417.

STACEY, K. A., SYMONDS, N. D. & ATKINSON, C. (1966). (In Press.)

WEINBERG, R. & LATHAM, A. B. (1956). Apparent mutagenic effect of thymine deficiency for a thymine requiring strain of *E. coli*. *J. Bact.* **72**, 570.

Antigenic Diversity of Mammalian Papillomaviruses

By G. L. LE BOUVIER,* M. SUSSMAN and L. V. CRAWFORD

M.R.C. Experimental Virus Research Unit, University of Glasgow

SUMMARY

Virions of the papillomaviruses of man, cattle, dog and rabbit were compared by immunodiffusion in agar. Antigen reactants were prepared from saline extracts of warts, and consisted either of crude virus-particle concentrates, or of 'full' or 'empty' fractions from equilibrium density gradients. There were no cross-reactions between the wart viruses of different species. Within any one species, 'full' and 'empty' particles gave antigenically identical single bands of precipitate, whether derived from the same or from different warts; but some old preparations of 'empty' particles gave a second band.

INTRODUCTION

Warts of broadly similar appearance and behaviour are found on the skin of men, dogs, cattle, cottontail rabbits, horses, and several other mammalian species (cf. Olson, 1963). From those of the first four, particles of the causative virus can be extracted which have morphologically identical capsids and contain DNA of molecular weight $\sim 5 \times 10^6$ (Crawford & Crawford, 1963; Crawford, 1965). These four kinds of papillomavirus (PV) do differ in host specificity, and show small differences in the guanine + cytosine content of their DNA (Crawford & Crawford, 1963). Conceivably, each virus originated independently in its particular host species. On the other hand, their striking resemblance in structure and activity could betoken a common ancestry. On the second hypothesis, a family relationship of this kind might be reflected in some degree of continuing homology between the genetic material of the four viruses, and also between their gene products. In the case of human and rabbit PVs, homology between the viral DNAs has not been detected (Crawford, 1965). The present note reports the dissimilar antigenic configuration of the virions of all four viruses.

METHODS

Antigen-antibody precipitation reactions were compared in two-dimensional, double-diffusion tests in agar, as described elsewhere (Martin *et al.* 1966); readings were made after 18–24 hr at room temperature.

Antigen reactants consisted of virus extracted from skin warts, and also, in the dog, from oral papillomata. Reactants were prepared in two ways: (i) crude 100-fold concentrates of virus were obtained by centrifuging virus particles from 10 to 20 % (w/v) saline homogenates of wart at 30,000 rev./min. for 2 hr in a Spinco Model L centrifuge; (ii) 'full' and 'empty' virus-particle fractions were obtained by centri-

fuging extracts of trypsinized warts in RbCl equilibrium density gradients, as described by Crawford & Crawford (1963).

Antibody reactants consisted of sera from rabbits immunized with 'empty' or 'full'+'empty' fractions of human, bovine, or rabbit PV. The rabbits usually received one intramuscular injection of virus in Freund's complete adjuvant followed by a second dose of virus in saline after 3–6 weeks, and were bled 7–10 days after the second injection. Sera from wart-bearing cows were also tested. For canine virus, the antibody reactant consisted of sera from dogs with regressing or recently regressed oral papillomata.

Aggregates of virus + antibody were examined in the electron microscope by the technique of negative staining with phosphotungstic acid described by Watson (1962).

RESULTS

Comparison of different papillomaviruses

The precipitation reactions of the four species of wart virus were quite distinct: a single band of precipitate was formed by homologous virus and serum reactants, but not by any of the heterologous combinations; nor were precipitins absorbed by any heterologous virus.

There was no suggestion of antigenic dissimilarity among different virus concentrates from any given species, including those prepared from a variety of bovine warts—American, French and Scottish—and those derived from oral and dermal warts of several dogs. Because sera from animals with warts might be more sensitive than hyperimmune antisera in detecting strain differences within any one species of PV, convalescent sera were used in some tests. Convalescent sera from 3 dogs had detectable precipitins, but revealed no antigenic difference between 4 specimens of canine virus, 1 from dermal and 3 from oral papillomata. Sera from 8 cows with 'angleberries' were negative, including 4 that had been injected with formolized preparations of their own warts. We tested no human sera for strain differences, but Almeida & Goffe (1965) reported positive precipitation reactions in agar with sera from patients: with a given virus concentrate, a single identical band was formed by different sera; with some sera a second fainter band was occasionally observed.

Control experiments

No precipitate was given with any of the 4 PV reactants by antisera from rabbits injected with the Toronto small-plaque strain of polyomavirus grown in secondary cultures of mouse-embryo cells. These anti-polyoma sera formed similar, and completely fusing, single bands of precipitate with 'full'- and 'empty'-particle fractions of polyomavirus separated in CsCl equilibrium density gradients. The polyomavirus fractions did not react with antibodies against any of the four PVs.

As a further control of specificity, the human-PV antiserum was pre-mixed with 20 % saline suspensions of cultured human skin cells, or with minced fresh skin from individuals of blood groups A, B and O, with or without prior exposure of the tissue to 0·25 % trypsin for 3 hr at 37°. Such pre-mixture failed to remove the antibodies reacting with antigens present in human wart virus concentrates. Analogous results were obtained on absorption of bovine-PV antiserum with fresh and cultured bovine skin.

Electron microscopy

Electron microscopic examination of mixtures of virus and homologous anti-serum in liquid showed clusters of virus particles, coated and linked together by filamentous molecules of antibody globulin, as first described by Almeida, Cinader & Howatson (1963). The specificity of the interaction of antigen with antibody was demonstrated by adding the slightly smaller polyomavirus to the suspension of PV: without antiserum, virus particles of both kinds were dispersed at random (Pl. 1, fig. 1); after admixture of antiserum, the aggregates were seen to consist wholly or predominantly of PV particles connected by antibody bridges (Pl. 1, fig. 2), with the unaggregated polyomavirus particles distributed around them (Pl. 1, fig. 3).
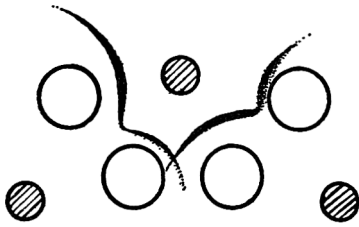
Fig. 1   Fig. 2

Figs. 1 and 2 are drawings of immunodiffusion tests
in agar read after 20 hr at room temperature.

Fig. 1. Absence of cross-reaction between human and rabbit PVs; but identity of reaction between 'full'- and 'empty'-particle fractions within each species. The four larger cups contain, from left to right: Shope rabbit PV, 'empty' fraction; Shope rabbit PV, 'full' fraction; human PV, 'full' fraction; human PV, 'empty' fraction. The three smaller cups contain, from left to right: human-PV antiserum; mixed human-PV and Shope-PV antisera; Shope-PV antiserum.

Fig. 2. Formation of 2 precipitate bands by some 'empty'-particle preparations of human PV. For reasons given in the Discussion section, the various bands have been designated α, β and γ. The α- and β-bands have not yet visibly fused. The upper two cups contain, from left to right: human PV, 'full'-particle preparation; and human PV, long-stored 'empty'-particle preparation. The lower cup contains human-PV antiserum.

Comparison of 'full' and 'empty' particles

'Full'- and 'empty'-particle fractions of the PVs of man, dog and rabbit were compared. For a given virus, the two fractions were antigenically indistinguishable by immunodiffusion, forming single bands of precipitate similar in character and showing complete fusion (Fig. 1). With bovine wart extracts, it was not possible to obtain well defined 'full' and 'empty' fractions, and both kinds of particles were tested as a combined reactant.

Three 'empty'-particle preparations of human PV, which had been stored at 4° for $1\frac{1}{2}$–2 years and then at −20° or −70° for a further 6–12 months, were found to form two specific bands of precipitate instead of the one which they had given when tested during the first month after extraction (Fig. 2). One of these two bands (β) often fused, after 2–3 days' development, with the single band (α) formed by other 'empty'-particle preparations or by 'full'-particle fractions; but it was usually less dense than the α-band, and further from the cup containing the virus reactant.

The second band (γ) developed still further from the virus cup, showed no obvious fusion with the α-bands of other preparations, but fused completely with the γ-bands of other long-stored 'empty' preparations of human PV. Despite the lack of a clear reaction of partial identity between α- and γ-precipitates, no γ-band was ever seen to cross an α-band; and virus concentrates which formed only α-precipitates did absorb small amounts of anti-γ antibody. One 'empty'-particle preparation of Shope rabbit PV likewise produced two immune precipitates, neither of which fused with the β- or γ-bands of human PV.

DISCUSSION

Although the single α-bands of precipitate formed in agar by PV concentrates and their homologous antisera were not themselves extracted and examined in the electron microscope, they probably represent aggregates of virions, like those seen when the same reactants are mixed in liquid. With polyomavirus, it has been shown that the similar precipitate band formed in agar is composed of virions linked by antibody bridges (Watson, Le Bouvier, Tomlinson & Walkey, 1966). One cannot yet say whether the antigen of the virus surface reacting with precipitin is formed by the structural protein of the capsid itself, by a virus-directed cellular neoantigen strongly adsorbed to capsid protein, or by a combination of both.

The two bands of precipitate observed with certain long-stored preparations of 'empty', and perhaps disintegrating, particles of human and rabbit PV seem to represent distinct antigenic components of the whole virion. They resemble in appearance and position the β- and γ-precipitates formed by polyomavirus reactants after treatment with trypsin (Le Bouvier, Watson & Thorne, in preparation); because of this resemblance, the different PV precipitates have for convenience been termed correspondingly α, β and γ (Fig. 2). However, these same 'empty' PV fractions, when tested soon after preparation, had formed only one band of precipitate, and this property had not, as in the case of polyomavirus, been altered by treatment with trypsin. Moreover, with bovine PV at least, trypsin treatment of untrypsinized virus concentrates did not, by itself, have any effect comparable to that of trypsin on polyomavirus: exposure to enzyme (0·13 % final concentration in Tris-buffered saline, pH 7·7) for 1½ hr at 37° increased the antigenic potency by two- to fourfold, but did not result in the formation of the γ-band.

Neither the DNAs nor the virion proteins of the wart viruses so far studied have given any evidence of a family relationship. It is still possible that signs of a common origin may be found in the substructure of the viral coat proteins, and ultimately in the amino acid sequence of their constituent polypeptides, rather than in their overall antigenic configuration as components of the intact capsid.

G. L. LE BOUVIER, M. SUSSMAN and L. V. CRAWFORD

Dr A. Girdwood Fergusson, Department of Dermatology, Stobhill Hospital, who provided human warts; Mr Brian Martin, BVMS, Kilmarnock, who provided bovine warts and sera; Dr George W. Crighton, University Veterinary Hospital, Glasgow, who provided bovine and canine warts and sera.

REFERENCES

ALMEIDA, J. D. & GOFFE, A. P. (1965). Antibody to wart virus in human sera demonstrated by electron microscopy and precipitin tests. *Lancet* ii, 1205.

ALMEIDA, J. D., CINADER, B. & HOWATSON, A. (1963). The structure of antigen-antibody complexes. *J. exp. Med.* 118, 327.

CRAWFORD, L. V. (1965). A study of human papilloma virus DNA. *J. mol. Biol.* 13, 362.

CRAWFORD, L. V. & CRAWFORD, E. M. (1963). A comparative study of polyoma and papilloma viruses. *Virology* 21, 258.

MARTIN, W. B., HAY, D., CRAWFORD, L. V., LE BOUVIER, G. L. & CRAWFORD, E. M. (1966). Characteristics of bovine mammillitis virus. *J. gen. Microbiol.* 45, 325.

OLSON, C. (1963). Cutaneous papillomatosis in cattle and other animals. *Ann. N.Y. Acad. Sci.* 108, 1042.

WATSON, D. H. (1962). Electron micrographic counts of phosphotungstate sprayed virus. *Biochim. biophys. Acta* 61, 321.

WATSON, D. H., LE BOUVIER, G. L., TOMLINSON, J. A. & WALKEY, D. G. A. (1966). Electron microscopy of antigen precipitates extracted from gel diffusion plates. *Immunology* 10, 305.

EXPLANATION OF PLATE

Figs. 1, 2 and 3 are electron-micrographs of mixtures of bovine PV and the somewhat smaller polyomavirus particles, obtained from RbCl equilibrium density gradients, and negatively stained with phosphotungstic acid. The magnification throughout is × 95,000.

Fig. 1. Without addition of antiserum: particles of both viruses are evenly dispersed.

Fig. 2. After addition of bovine-PV rabbit antiserum: antibody bridges link the aggregated PV particles.

Fig. 3. After addition of antiserum: note the aggregate of PV particles, with one polyomavirus particle entrapped (indicated by arrow), and others distributed at random.

503

Biosynthesis of the Antibiotic Nisin and Other Basic Peptides by *Streptococcus lactis* Grown in Batch Culture

By A. HURST*

Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford

SUMMARY

Streptococcus lactis was grown in batch culture in a complex organic medium. Growth of the culture was followed by estimating the extinction, the dry weight, protein, DNA and RNA of the organism; basic peptides were estimated by electrophoresis and nisin by bio-assay. By the end of the lag phase of growth when active DNA and RNA synthesis were already proceeding, the nisin which had been introduced with the inoculum could not be recovered, although the cocci contained other basic peptides. Rapid nisin synthesis started after an increase of about 50 % in the dry weight of the cocci had taken place. Initially, high molecular weight nisin was made, the concentration of which decreased as the incubation proceeded. This was followed by the production of lower molecular weight nisin which could be recovered 24 hr after the end of the incubation. Inocula of organisms derived at intervals from the parent culture were tested in secondary cultures for length of lag of growth by re-inoculation into fresh medium of the same composition. During the lag phase of growth of the parent culture, the length of lag of growth of the secondary cultures fell to about half the original time. The shortest lag of the secondary cultures coincided with commencement of logarithmic growth of the parent culture and with the disappearance of cellular nisin from the parent culture. The length of the lag of the secondary cultures returned to their original value as the parent culture reached stationary phase and coincided with maximum nisin concentration in the parent culture. It is suggested that the length of lag of growth is related to the presence of basic peptides.

INTRODUCTION

A variety of micro-organisms produce antibiotic peptides, but the function of these substances to the organism producing them is unknown. Katz, Wise & Weissbach (1965) speculated that the synthesis of actinomycin might represent terminal growth processes. Bernlohr & Novelli (1963) were able to feed doubly labelled bacitracin to cultures of *Bacillus licheniformis* and recover the antibiotic in an apparently unchanged condition from the spores; they concluded that it might form a subunit of the spore coat. On the other hand, Brenner, Gray & Paulus (1964) were unable to recover polymyxin from the spore coat of *B. polymixa* and concluded that polymyxin, if present, was less than 0·2 % of the dry weight of the spore. Woodruff (1966) believed that antibiotics are waste products of the cell formed at a time when an essential metabolite becomes limiting.

* Formerly A. Hirsch.

Halvorson (1965) suggested that polypeptide antibiotics may play a part in the differentiation of the vegetative bacterium by repressing the vegetative cell genome. This suggestion is supported by the results of Spizizen (1965) who found that asporogenous mutants of bacilli also failed to produce an 'antibacterial factor'. Szabo, Vitalis & Bekesi (1966) obtained a polypeptide from a conidia forming Streptomyces which restored the conidial forming ability of a mutant which ceased to form them; the polypeptide appeared to stabilize messenger RNA. Moore & Shockman (1966) obtained a basic polypeptide from *Streptococcus faecalis* which inhibited protein synthesis in a cell-free system prepared from the same organism; this inhibitor appeared to be associated with RNA. Hurst & Taylor (1965) were able to isolate, in three out of seventeen attempts, basic peptides from *Escherichia coli* which inhibited the growth of the organism from which these substances were isolated.

Washed suspensions of *Streptococcus lactis* synthesized nisin by a mechanism which was similar to that of protein synthesis, in that it was sensitive to inhibition by chloramphenicol and puromycin but differed by being more sensitive to these antibiotics and synthesis was always preceded by a delay (Hurst, 1966). Growing cultures also show a delay of synthesis of nisin since this did not start till the logarithmic phase of growth (Hirsch, 1951). In this respect nisin synthesis resembles that of other polypeptide antibiotics (for review see Abraham, Newton & Warren, 1965). This differential synthesis, if confirmed, could suggest that the basic peptides and nisin had a possible function in the life-cycle of the organism. The present paper describes experiments to examine this and relates nisin biosynthesis to other biochemical events which occur during the growth of the organism.

METHODS

Organisms, media and culture conditions were described before (Hurst, 1966) For cultivation experiments the nisin producing *Streptococcus lactis* was subcultured twice at 24 hr intervals and then used as inoculum for batch culture. Immediately before inoculation the culture was diluted with an equal volume of medium 22 prewarmed to 30° and incubated for 1 hr at 30°; the volume was adjusted to give a 5 % (v/v) inoculum. Incubations were usually done in 20 l. volumes and no attempts was made to stir, or to control the pH value or oxygen tension.

Preparation of samples and estimations

Length of lag of growth. Samples of the culture were taken at intervals during incubations to estimate the length of the lag time of the culture at different stages of the growth cycle. The samples were cooled in crushed ice and the cocci centrifuged off in the cold. They were taken up in chilled distilled water and the extinction (E) adjusted to correspond to 6–8 mg. dry wt. organism/ml. This was then diluted 1/100 in medium 22 at 30° and incubated in a 30° water bath. The moment when the cold culture was diluted with the warm medium was taken as time zero. At intervals the extinction of this secondary culture was determined and the results were plotted as a series of curves of extinction against time. A tangent was drawn through the lag-phase points of the curves and another tangent through the logarithmic phase points; the intersect of the tangents was read off on the time scale and was the estimated length of lag in minutes (Monod, 1949; Meynell & Meynell, 1965).

Extinctions were measured on an S.P. 600 spectrophotometer at 600 mμ in 1 cm. cuvettes (Cambridge Instrument Co.).

Nucleic acids and protein. Reaction was stopped by diluting samples with an equal volume of cold N-perchloric acid (PCA). After two washes with cold 0·5 N-PCA the nucleic acids were extracted 3 times with 0·5 N-PCA at 70° for 30 min. The residue was dissolved in 2N-NaOH and was used for estimating protein by the method of Lowry, Rosebrough, Farr & Randall (1951). The supernatant fluid was used to estimate DNA by the diphenylamine method (Burton, 1956) and RNA by the orcinol method (Mejbaum, 1939).

Nisin (free and bound = total nisin) was estimated by the modified method of Berridge & Barrett as previously described (Hurst, 1966). For estimating nisin bound to the organisms (cellular nisin) the reaction was stopped by chilling, the organisms centrifuged down in the cold and the supernatant fluid replaced by an equal volume of 0·05 N-HCl in which the cocci were resuspended. The nisin was then extracted by placing the tubes in a boiling water bath for 5 min. and the extracts assayed as before against *Streptococcus cremoris* (strain 1P5).

Preparation of basic peptides and nisin. Treatment as described above with hot 0·05 N-HCl extracted nucleic acids, basic peptides and nisin. The nucleic acids were not examined and were lost in the purification steps. Nisin and basic peptides which might have been in solution also were not examined. The method used aimed to estimate the qualitative and semi-quantitative changes which occurred in the cells during a growth cycle and was as follows. During the early phases of growth when the concentration of organisms was only 100–200 μg. dry wt./ml., 3–4 l. of culture medium were chilled by mixing with crushed distilled water ice and the cocci centrifuged down in the cold. As growth proceeded and the concentration of cocci increased, 1 l. samples were sufficient; these could be chilled quickly by immersion into crushed ice. In this way the quantity of cocci for each preparation was kept approximately constant and enabled semi-quantitative comparison to be made of the final preparations, irrespective of the original concentration of cocci in the culture. The cocci so obtained were washed once with cold 0·1 M-phosphate buffer (pH 7) and then 1 g. wet wt. cocci were taken up in 10 ml. 0·05 N-HCl, placed in a boiling water bath for 5 min., cooled, centrifuged and the deposit discarded. The basic peptides were concentrated into *n*-propanol by the method of Cheeseman & Berridge (1957); each 100 ml. of solution in 0·05 N-HCl was shaken up with 15 ml. *n*-propanol, followed by 21 g. NaCl, shaken and then left to stand, This operation was not repeated. The propanol layer and the interface beneath it were pipetted off and evaporated in a rotary evaporator to near dryness or were freeze dried. The dry material was then made up to 15 ml. with 5 % (v/v) acetic acid and desalted through a 1 × 15 cm. column of Amberlite resin CG 50 (British Drug Houses Ltd., Poole, Dorset) finished in the H$^+$ form (Dixon, 1959). The column was washed with 5 % (v/v) acetic acid and the basic peptides eluted with 50 % acetic acid. Figure 1 shows the elution histogram of nisin. The active fractions were combined and freeze-dried.

Electrophoresis. The freeze dried powders were made up as 1 % (w/v) solutions in 0·05 N-HCl. The solutions were run in polyacrilamide gels prepared by the method of Tombs (1965); 20 % (w/v) gels were made up in distilled water and after setting were equilibrated for 1 day with a pH 2 'buffer' made up of 11·5 ml. formic acid,

1500 ml. glycerol and distilled water to 5 l. Purified nisin (kindly provided by Dr J. Tramer, United Dairies Central Laboratories, London) and polymyxin (Burroughs Wellcome Ltd., London) in a mixture each at 1 % (w/v) in 0·05 N-HCl were used as markers. The electrophoresis was run in the same buffer at 12·5 V/cm. and 0·5 mA for 4–6 hr. The gels were stained for 5 min. with naphthalene black and decolorized by washing with distilled water.

Bands of growth inhibitory substances were identified by placing the gels on agar plates seeded with *Streptococcus cremoris*. To prevent inhibition due to acidity the gels were first neutralized by washing in the cold with 0·05 M-tris buffer (pH 8). The gel was then dried with cellulose tissue and placed on the surface of a yeast glucose agar plate inoculated 1 % (v/v) of the culture nisin bioassay test-organism. Alternatively, two gels were run simultaneously; one was then stained and the other was neutralized. The stained gel was used to guide the slicing of the unstained but
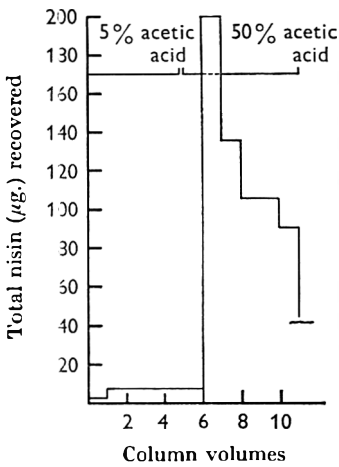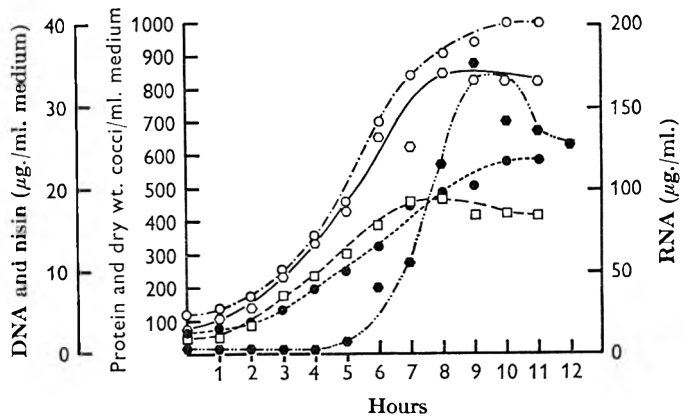
Fig. 1   Fig. 2

Fig. 1. Elution histogram of nisin from Amberlite Resin CG 50.
Fig. 2. Biosynthetic activities of *Streptococcus lactis* growing in batch culture. Dry weight, ○—·—; DNA, ○———; Nisin, ●—··—; Protein, ●‑‑‑; RNA, □———.

neutralized gel; the slices of gel were then placed on the seeded agar plates.

After contact for 1 hr the gel was removed and the plate incubated overnight at 30°; because of the high concentrations needed for electrophoresis, contact for longer than 1 hr could completely clear the agar plate; contact for less than 1 hr was not tried.

RESULTS

Characteristics of the growth of Streptococcus lactis

The composite data in Fig. 2 are based on four separate experiments and show the sequence of changes taking place during batch culture. Comparison between experiments showed that the length of the lag varied, the slope of the growth curve varied and the rate at which the nisin was formed during the experiment and subsequently disappeared also varied. However, these differences were considered to be

inherent in experiments done with different inocula, in different batches of media and were not investigated further.

Figure 2 shows that during the first 2 hr the dry weight and protein of the organism increased less than DNA and RNA. The DNA and RNA synthesis attain maximum rate before protein synthesis and dry weight increase. The small amount of nisin introduced with the inoculum decreased during the lag phase. Total nisin was
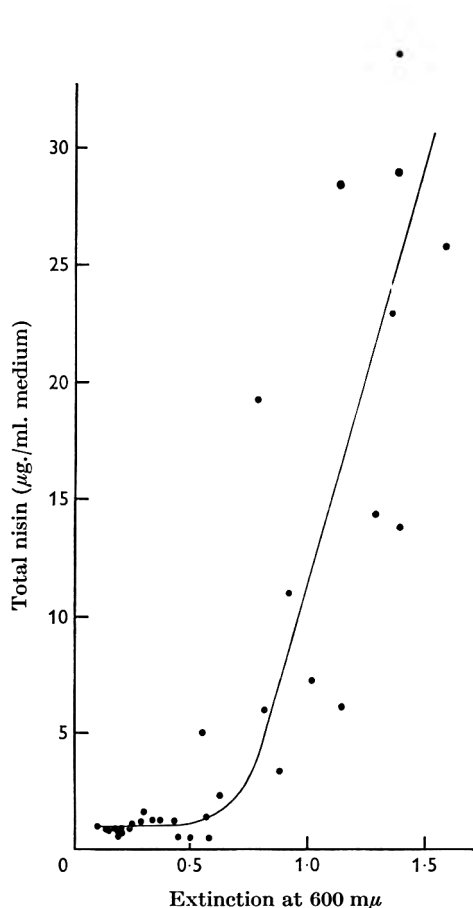
Fig. 3
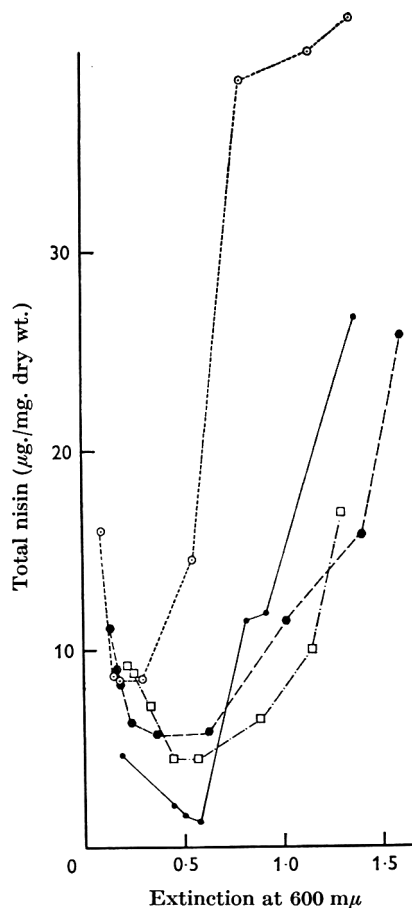
Fig. 4

Fig. 3. Growth of *Streptococcus lactis* and differential synthesis of nisin.

Fig. 4. Growth of *Streptococcus lactis* and differential synthesis of nisin in four different fermentation experiments. Expt. 1, ⊙, – – –; Expt. 2, ●———; Expt. 3, ◆ — — —; Expt. 4, □—·—·—.

maximal at 9 hr after which it decreased. From Fig. 2 it can be seen that nisin and protein synthesis occurred at different rates, Fig. 3 is a differential plot made according to Monod, Pappenheimer & Cohen-Bazier (1952); the extinction was the measure usually used and has been used in Fig. 3, which presents all the data obtained in four separate experiments over 5 months. Figure 3 shows that nisin synthesis did not occur during the early stage of the growth when the extinction

was low. Its rate increased sharply after considerable growth had already taken place. Figure 4 is based on the same data as Fig. 3 but the data plotted are μg. total nisin/mg. dry wt. against the extinction. In Fig. 4 the results of the four experiments which were made on separate occasions can no longer be superimposed and are shown separately. Not only is the differential rate of nisin synthesis evident but a new phenomenon is seen, namely that the nisin introduced with the inoculum was so modified that it was not detected by the bio-assay.

The data of Figs. 2–4 referred to total nisin; Fig. 5 is the record of an experiment especially designed to follow cellular nisin during the early parts of the incubation. It confirms the suggestion of Fig. 4 that cellular nisin was first destroyed before active synthesis began.
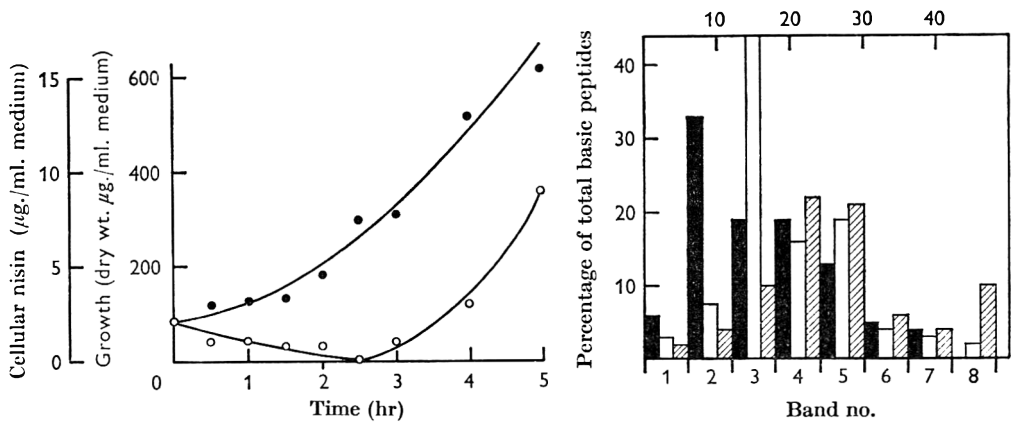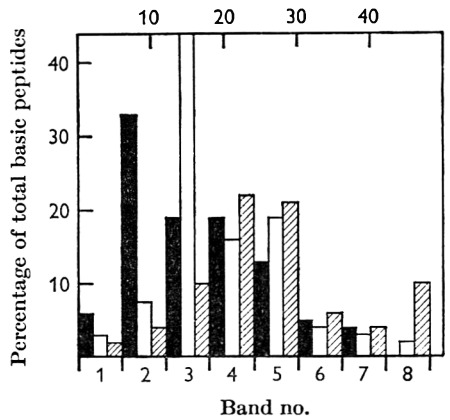
Fig. 5. Initiation of growth of *Streptococci lactis* and synthesis and disappearance of cellular nisin. Growth, ●———; cellular nisin, ○———.

Fig. 6. Percentage distribution of different basic peptides in cultures of *Streptococcus lactis*, 3, 7 and 15 hr old. Culture age 3 hr, ▉; culture age 7 hr, □; culture age 15 hr, ▨. Band seven corresponds to high mol.wt. nisin (slow nisin). Band eight corresponds to nisin.

Electrophoresis of the basic peptides formed at different stages of growth. The markers, nisin and polymyxin were well separated, the lower molecular weight polymyxin being about 25 mm. in front of nisin. Frequently, the purified nisin was split into two components, a small amount of slower moving material ('slow nisin') and a main component which was faster moving but was, nevertheless, well behind polymyxin.

Preparations made from 3, 7 and 15 hr cultures revealed up to eight bands of basic peptides. Band 1, the highest molecular weight material remained near the origin; band 7 corresponded to 'slow nisin' and band 8 to the faster moving main nisin component. Bands 2–6 occupied an intermediate position. Band 8 (main nisin component) was absent in the preparation made from the 3 hr culture. Preparations made from 7 hr cultures contained a considerable amount of slow-moving bands 2–6 material, 'slow nisin' (band 7) and traces of nisin (band 8). Preparations made from 15 hr culture contained more of band 8 than band 7 and the intensity of bands 2–6 diminished.

These results can be expressed quantitatively as shown in Fig. 6. Photographs of gels were examined in an integrating densitometer ('Chromoscan', Joyce Loebl and Co., Newcastle on Tyne); each area of each preparation was taken as 100 % and the eight bands present in each preparation were expressed as a percentage of the total area of that preparation.

Impressions of gels on seeded agar plates showed that only the faster bands were inhibitory (bands 7 and 8). Bands 2–6 were not inhibitory. Occasionally band 1 (origin) was also inhibitory. Since the front of the 3 hr. preparation and that of the 15 hr preparation did not correspond, this was additional evidence that at least two inhibitors were present in the 15 hr preparation, but only traces of one in the 3 hr. preparation. Both bands of the marker nisin appeared to be inhibitory. Polymyxin did not inhibit the test-organism *Streptococcus cremoris*.
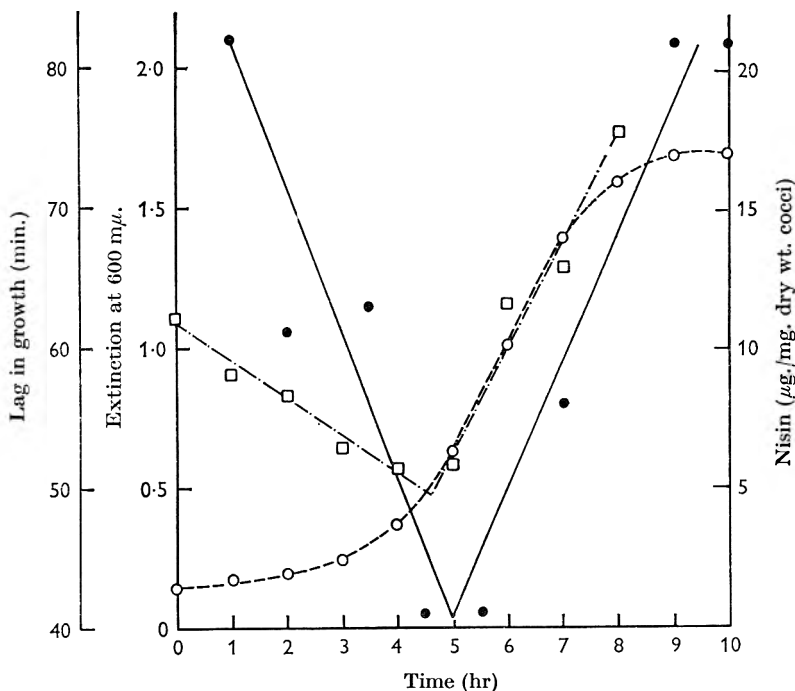
Fig. 7. Growth, synthesis of total nisin and length of lag of a culture of *Streptococcus lactis*. Minutes of lag of growth, ●————; extinction ○— — —; total nisin, □—·—·—.

Length of lag phase of growth

Inocula made from the culture at different stages of the growth cycle showed that the lag decreased and then increased. The results of a typical experiment are shown in Fig. 7. Samples taken during the early part of the incubation gave a lag of 82 min.; this decreased to 41 min. at the point when the culture entered the logarithmic growth phase; the lag of growth rose again to 82 min, with samples from the culture entering the stationary phase. Figure 7 also shows that the concentration of nisin (μg./mg. dry wt. cocci) followed a similar variation with time. This observation was repeated in three independent experiments.

Description of the biochemical events which occur during growth

DNA and RNA synthesis began during the lag period of growth (Fig. 2). At the same time the nisin introduced with the inoculum gradually diminished and at $2\frac{1}{2}$ hr no nisin was recovered from the cocci (Fig. 5). Electrophoresis showed that 3 hr cocci had little or no nisin corresponding to that which was the main component of 15 hr cocci, although they contained considerable amounts of other basic peptides. When the cocci were harvested at intervals and used as inocula to initiate new growth the lag period of growth first decreased and then increased while the parent culture was in the late logarithmic phase of growth. The minimum and maximum lag periods of growth and the mimimum and maximum nisin concentrations almost coincided (Fig. 7).

During the logarithmic phase of growth, RNA, DNA and protein increased at about the same relative rates. Rapid nisin synthesis started after the concentration of organisms as dry wt, had increased by 50 % of the total attained in 12 hr (Figs. 2, 3). Electrophoresis showed that 7 hr cocci had all the basic peptide components of 15 hr cocci but in different proportions. The nisin had two components; the slower moving band corresponded to the main nisin component of 7 hr cocci which had little of the faster moving component. This situation was reversed in the preparations made from 15 hr cocci (Fig. 6.)

The stationary phase of growth started by the halting of the synthesis of DNA, RNA, protein and nisin (Fig. 2). The electrophoretic pattern of preparations made from 24 hr cocci was similar to that obtained from 15 hr cocci.

DISCUSSION

It was suggested (Hurst, 1966) that nisin synthesis resembles protein synthesis. Cheeseman & Berridge (1957) suggested that the molecular weight of nisin was 7000; this puts it in the class of small proteins. Bodansky & Perlman (1964) suggested that the molecular weight of nisin was more likely to be one-third of the figure given by Cheeseman & Berridge. However, the clear separation of nisin from polymyxin (which has molecular weight of 1250) and its lack of non-specific diffusion in polyacrilamide gels suggest that the original estimate of Cheeseman & Berridge is more likely to be correct. The synthesis of nisin is clearly differentiated from that of other intracellular proteins; during early growth nisin synthesis does not take place, and this delay in synthesis is similar to that previously observed with washed organisms (Hurst, 1966). Figure 3 also resembles previously published figures for the induced synthesis of β-galactosidase by *Escherichia coli* (Monod *et al.* 1953, Nakada & Magasanik, 1964; Moses & Calvin, 1965). *Streptococcus lactis* also appears to have an inducible β-galactosidase system (Citti, Sandine & Elliker, 1965). Perhaps the delay in nisin synthesis can be explained by a similar inductive control.

It is not known how *Streptococcus lactis* inactivates the nisin it produces before growth commences. Many bacterial species are known which inactivate nisin (Alifax & Chevallier, 1962) but it was not previously known that the organism which produces nisin also inactivates it at two distinct periods of its growth in batch culture. The first of these points occurs during the lag phase of growth and might be explained either by the presence of an inactivating system or by multiplication of cocci which

do not have nisin or both. A nisin-inactivating system seems more likely because the total nisin, not only cellular nisin, declined. Secondly, nisin destruction is indicated by the peak (Fig. 2) which occurred just before the onset of stationary phase of growth and which was consistently observed in four separate experiments. It can be explained by the disappearance of the 'slow nisin' (band 7) as the culture aged. This was replaced by the faster moving nisin which remained stable for at least 24 hr; the cause of the instability of the slow band is not known. At least four different nisins are known to exist (Berridge, Newton & Abraham, 1952).

Sonneborn (1963) stated that cell differentiation is associated with division but can also occur in the absence of division as cells grow older. It is difficult to tell which of these processes occurs in a growing bacterial culture. Jacob & Monod (1963) defined differentiation as follows: 'Two cells are differentiated with respect to each other if, while they harbour the same genome, the patterns of proteins which they synthesize is different.' This is the process which has been demonstrated to occur with respect to nisin and other basic peptides when *Streptococcus lactis* grows in a batch culture. It is tempting to think that each individual in the population is preparing first for active growth by the destruction of the nisin which it carries, and at the end of the growth is preparing for dormancy by producing nisin. It is a classic observation of bacteriology that the resistance of cells to unfavourable physical conditions, salt, heat, irradiation, germicides, etc., vary with the growth phase (Porter, 1946). It is possible that nisin acts as a suppressor of a part of the genome concerned with rapid multiplication and enables the cell to synthesize more resistant systems. This suggestion is similar to that made by Halvorson (1965) for the function of peptide antibiotics in sporeforming bacteria. The suggestion made earlier that nisin synthesis is under inductive control also fits the bacillus antibiotic peptides (Srinivasan 1966). Recently it has been suggested that the whole sporulation process is connected with catabolic repression (Schaeffer, Millet & Aubert, 1965). It follows from these suggestions that growth rate and initiation of growth (length of lag) may be two distinct processes, as has been shown in this paper. It is not the first time that such results have been reported and indeed, about 30 years ago this was the currently accepted concept (for review see Porter, 1946). Chesney (quoted by Porter, 1946) working with type I Pneumococcus, used a lag technique very similar to that described in this paper and obtained similar results. He regarded the occurrence of lag as 'an expression of injury which the bacterium has sustained from its previous environment'. The toxic agent in this case was later thought to be hydrogen peroxide. Since most microbes do not accumulate H_2O_2, Chesney's explanation fell into disrepute. However, the suggestion put forward in this work is that the length of lag of growth might be controlled by the presence of basic peptides.

REFERENCES

ABRAHAM, E. P., NEWTON, G. G. F. & WARREN, S. C. (1965). Problems relating to the biosynthesis of peptide antibiotics. In *Biogenesis of Antibiotic Substances*. Ed. by Z. Vanek and Z. Hostalek, p. 169. London: Academic Press.

ALIFAX, M. & CHEVALLIER, M. (1962). Étude de la nisinase produite par *Streptococcus thermophilus*. *J. dairy Res.* **29**, 233.

BERRIDGE, N. J., NEWTON, G. G. F. & ABRAHAM, E. P. (1952). Purification and nature of the antibiotic nisin. *Biochem. J.* **52**, 529.

BERNLOHR, M. W. & NOVELLI, G. M. (1963). Bacitracin biosynthesis and spore formation: The physiological role of an antibiotic. *Archs Biochem. Biophys.* **103**, 94.

BRENNER, M., GRAY, E. & PAULUS, H. (1964). The relation of polymyxin B to the spore coat of *Bacillus polymyxa*. *Biochim. biophys. Acta* **90**, 401.

BODANSKY, M. & PERLMAN, D. (1964). Are peptide antibiotics small proteins? *Nature, Lond.* **204**, 840.

BURTON, K, (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315.

CHEESEMAN, G. C. & BERRIDGE, N. J. (1957). An improved method of preparing nisin. *Biochem. J.* **65**, 1957.

CITTI, J. E., SANDINE, W. E. & ELLIKER, P. M. (1965). β-galactosidase of *Streptococcus lactis*. *J. Bact.* **89**, 937.

DIXON, H. F. B. (1959). A method of desalting certain polypeptides. *Biochim. biophys. Acta* **34**, 251.

HALVORSON, H. O. (1965). Sequential expression of biochemical events during intracellular differentiation. *Symp. Soc. gen. Microbiol.* **15**, 343.

HIRSCH, A. (1951). Growth and nisin production of a strain of *Streptococcus lactis*. *J. gen. Microbiol.* **5**, 208.

HURST, A. (1966). Biosynthesis of the antibiotic nisin by whole cells of *Streptococcus lactis*. *J. gen. Microbiol.* **44**, 209.

HURST, A. & TAYLOR, D. R. (1965). Growth inhibition of *Escherichia coli* by some basic peptides prepared from the same strain. *Nature, Lond.* **207**, 438.

JACOB, F. & MONOD, J. (1963). Genetic repression, allosteric inhibition and cellular differentiation. In *Cytodifferentiation and macromolecular synthesis*. Ed. by M. Locke, p. 30. London: Academic Press.

KATZ, E. WISE, M. & WEISSBACH, H. (1965). Actinomycin biosynthesis. Differential effect of chloramphenicol on protein and peptide antibiotic synthesis. *J. biol. Chem.* **240**, 3071.

LOWRY, O. H., ROSEBROUGH, H. J., FARR, A. L. & RANDALL, M. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MEJBAUM, W. (1939). Über die Bestimmung kleiner Pentosemengen insbesondere die merivaten der Adenylesaure. *Hoppe-Seyler's Z. physiol. Chem.* **258**, 117.

MEYNELL, G. G. & MEYNELL, E. (1965). *Theory and Practice in Experimental Bacteriology*. Cambridge University Press.

MONOD, J. (1949). The growth of bacterial cultures. *Rev. Microbiol.* **3**, 371.

MONOD, J., PAPPENHEIMER, A. M. JUN. & COHEN-BAZIRE, G. (1952). La cinétique de la biosynthese de la β-galactosidase chez *E. coli* considerée comme fonction de la croissance. *Biochim. biophys. Acta* **9**, 648.

MOORE, L. D. & SHOCKMAN, G. D. (1966). Accumulation of an inhibitor of protein synthesis after amino-acid deprival. *Fed. Proc.* **25**, 582.

MOSES, V. & CALVIN, M. (1965). Lifetime of bacterial messenger ribonucleic acid. *J. Bact.* **90**, 1205.

NAKADA, D. & MAGASANIK, B. (1964). The roles of inducer and catabolite repressor in the synthesis of β-galactosidase by *Escherichia coli*. *J. mol. Biol.* **8**, 105.

PORTER, G. (1946). *Bacterial Chemistry and Physiology*. London: Chapman and Hall.

SCHAEFFER, P., MILLET, J. & AUBERT, J. P. (1965). Catabolic repression of bacterial sporulation. *Proc. natn. Acad. Sci., U.S.A.* **54**, 704.

Sonneborn, T. M. (1963). The differentiation of cells. In *The Scientific Endeavour*, p. 217. NewYork: Rockefeller Institute Press.

Spizizen, J. (1965). Analysis of asporogenic mutants in *Bacillus subtilis* by genetic transformation. In *Spores III*. Ed. by L. L. Campbell and H. O. Halvorson, p. 125. American Soc. Microbiol.

Srinivasan, V. M. (1966). Sporogen—an 'inducer' for bacterial cell differentiation. *Nature, Lond.* 209, 537.

Szabo, G., Vitalis, S. & Bekesi, I. (1966). Endogenous factor in differentiation of *Streptomyces griseus*. *J. gen. Microbiol.* 44, v.

Tombs, M. P. (1965). The interpretation of gel electrophoresis. *Anal. Biochem.* 13, 121.

Woodruff, H. B. (1966). Physiology of antibiotic production: the role of the producing organism. *Symp. Soc. gen. Microbiol.* 16, 22.

Regeneration of Mycelial Protoplasts of *Fusarium culmorum*

By I. GARCÍA ACHA, F. LÓPEZ-BELMONTE
AND J. R. VILLANUEVA

Instituto de Biologia Celular, CSIC, Madrid-6, Spain

SUMMARY

The process of regeneration of *Fusarium culmorum* protoplasts, obtained by means of strepzyme RA, has been followed. Protoplast regeneration started with the formation of cellular aggregates originating a mass of globular forms which later gave rise to the formation of a pseudomycelial form. Regeneration of protoplasts was formed in three different ways: (*a*) by means of a chain of yeast-like forms and later originating a germ tube; (*b*) by direct formation of the germ tube from the protoplast; (*c*) through a complicated process with the formation of various globular forms and giving rise to the formation of the germ tube at the end. Maximum regeneration, about 80 % of the protoplasts, was found in the presence of 2 % (w/v) sucrose (or 1 % (w/v) glucose + 1 % (w/v) fructose) + 10 % (w/v) sorbose + 0·2 % agar + the mineral salts of the Czapek medium. No other sugars were able to substitute for sucrose and sorbose. Agar was the best substance for regeneration; gelatin produced an inhibition. Regeneration was greater under acid conditions, alkaline pH values interfering with the phenomenon. The number of nuclei/protoplast varied from 1 to 4, the lack of them in some spherical forms perhaps being the cause of failure to regenerate. No differences were found in the regenerative process as between protoplasts obtained by the use of snail or by microbial lytic enzymes.

INTRODUCTION

Studies of regeneration have been made with the cytoplasmic particles and protoplasts obtained from bacterial and yeast protoplasts obtained by different mechanical and enzymic methods. Regeneration of mould protoplasts was mentioned in the work of Emerson & Emerson (1958) and of Bachman & Bonner (1959) with *Neurospora crassa* protoplasts obtained by the action of a hemicellulase or by using a snail enzyme (*Helix pomatia*). Strunk (1965) reported regeneration in protoplasts of *Polystictus versicolor* and Villanueva (1966) in other fungi.

In the present work an attempt was made to examine some of the factors which affect the regeneration process in *Fusarium culmorum*; the naked protoplasts were characterized by their osmotic fragility and loss of the rigidity characteristic of the mycelial cells. These protoplast bodies appeared to have the same synthetic abilities as whole cells from which they were derived, including the ability to make a new wall.

METHOD

Preparation of protoplasts for regeneration studies. Protoplasts of mycelium of *Fusarium culmorum* (CECT, no. 2148) were obtained as described by Rodriguez Aguirre *et al.* (1964), by using a lytic system produced by *Streptomyces* strain RA.

Protoplasts were freed from mycelial debris by passing the suspension through a 11G2 Jena fritted glass filter, without pressure. Pure preparations of protoplasts were washed 3 times with the phosphate+citrate buffer (0·1 M, pH 6·5)+0·6 M-mannitol or NH_4Cl, centrifuged at 4000 g for 15 min., and the pellet resuspended and plated on media containing various supplements. The determination of reversion was made by phase-contrast microscopy in wet mount preparations and in hanging drops containing isolated protoplasts.

Determination of viability. The numbers of living and dead protoplasts were obtained by the methylene blue vital staining technique in which dead organisms stain blue while living organisms remain colourless. Viable counts were made by plating suitable dilutions in appropriate media.

Nuclear staining and microscopic examination. Protoplast suspensions and intact mycelium were stained with alkaline Nile Blue A by a modification of the method of Gancevici, Stoian & Keller (1962). A drop of the protoplast suspension previously washed with the stabilizer solution was placed on a microscope slide, mixed with 1 drop of 0·1 M-glycine buffer (pH 10) and 1 drop of Nile Blue A solution (0·02 %, w/v) and then examined under a coverslip with the phase-contrast microscope. This technique clearly showed a light purple nuclear body and a blue-green cytoplasm with orange lipid inclusions.

Attempts were also made to differentiate nuclei by the use of acridine orange solution (1/100,000) in the fluorescence microscope. The nuclei were stained bright green and the cytoplasm appeared yellow-green.

RESULTS AND DISCUSSION

Microscopic observations on protoplasts; number of nuclei

In *Fusarium culmorum*, by the use of the strepzyme preparation from *Streptomyces* RA, the hyphal content between two septa is liberated into one or more protoplasts (Villanueva, 1966). Thus, one protoplast does not always correspond to a single cell (López-Belmonte, García Acha & Villanueva 1966). Not all the protoplasts formed from the mycelium are similar in size. Their contents varies; the number of nuclei, vacuoles and cytoplasmic particles may be quite different (García Acha, López-Belmonte & Villaneuva, 1966).

According to our observations the number of nuclei between two septa is not constant in the hypha of *Fusarium culmorum*. The usual number is between 2 and 4. Nuclei can pass together with the cytoplasmic contents through the septa as we have seen during the liberation of protoplasts. The number of protoplasts liberated from one hyphal compartment is not constant, so that the number of nuclei/protoplast differs. Attempts to detect nuclei in protoplasts have sometimes failed. They can often be demonstrated by the specific staining techniques used, but the possibility cannot be excluded that they are not observed although they are present in the body. Most of the protoplasts possess at least one or two nuclei; protoplasts

with three nuclei have been observed. The size of a protoplast is not related to the number of nuclei in it. The importance of nuclear material has always been emphasized in work on regeneration of plant cells and of protozoa. We have suggested that only protoplasts possessing at least one nucleus are able to regenerate cell wall, giving rise to normal mycelial forms, what is in accordance with the results described by Rost & Venner (1965).

The process of regeneration; changes in morphology

The various stages of the regeneration process of *Fusarium culmorum* protoplasts were observed. For 8 hr there was no change in appearance; then the majority of the protoplasts began to develop a protrusion which increased in volume. Some protoplasts formed a large vacuole. Later, the complete regeneration of the abnormal forms into normal mycelium occurred. The earliest visible alteration in the morphology of the protoplasts was the formation of cellular aggregates. Some protoplasts divided and grew during the first 24 hr, giving more or less pseudomycelial figures with a wide variety of shapes. Other protoplasts developed a kind of spherical structure, not sensitive to osmotic shock, which was able to form hypha. We have observed at least three different ways to start regeneration of protoplast. (*a*) The protoplast gives a series of yeast-like forms grouped in a chain (Pl. 1, fig. 1). Some of these forms differentiated, becoming more refringent than their neighbours and finally producing a germ tube capable of more or less normal hypha-like growth. (*b*) The protoplast itself germinated to give a hyphal tube; but this was very uncommon. Individual protoplasts sometimes produced one or more hyphae as direct outgrowths of the mother cell (Pl. 1, fig. 2). (*c*) The original protoplast, keeping its spherical shape, produced a protrusion which enlarged, giving another spherical body which remained beside the first. Sometimes one of these bodies gave another body of ameboid form and the contents of the previous one were transferred to the neighbouring cell (Pl. 2, fig. 3). The remains of these shells (cell membranes or cell walls) can be seen in the preparation when the protoplasts grew to a mass of more or less bud-like and spherical forms, some with large refringent vacuoles, and some forming germ tubes to give normal mycelium. Plate 3, figs. 4 and 5 show examples of shell liberation.

It was also observed that the empty shells were sometimes filled again, so that the cytoplasmic material moved from one side to another leaving empty spaces during this transfer. The meaning of this phenomenon is unknown.

Emerson & Emerson (1958) described reversion of neurospora protoplasts upon transfer to media lacking the enzymes used for the digestion of the cell wall. Complete reversion to typical mycelial growth required from a few hours to several days and the type of mycelium formed seemed to depend upon the osmotic strength of the medium. Similar observations were made by Bachman & Bonner (1959) who stated that some of the protoplasts of *Neurospora crassa* obtained with the snail lytic enzyme system, when transferred to a suitable liquid or solid nutrient medium regenerated to give normal mycelial growth. The course of regeneration varied greatly.

Our results with protoplasts of *Fusarium culmorum* were more or less in agreement with these observations. Mycelium originated from an isolated protoplast, after transfer to fresh nutrient medium was able to sporulate normally. These

reversion cultures were indistinguishable from the original culture in their growth habit, morphology, chemical composition, sensitivity to strepzyme RA, and productivity of protoplasts. This means that a normal mycelium can originate from a protoplast absolutely devoid of cell wall. There is no doubt that the mycelial protoplasts from *Fusarium culmorum* are true naked protoplasts since the cell wall remains in the preparation, empty but keeping its shape, once the protoplasts have been released (Pl. 3, fig. 6). Electron microscopic studies of ultrathin sections of naked fusarium protoplasts showed no remnants of cell wall on the surface of the cytoplasmic membrane (to be published). As a consequence it can be concluded that on regeneration of these protoplasts thick cell walls are formed, suggesting *de novo* synthesis of the rigid envelope. The presence of some vesicles observed in the inner side of the plasma membrane of the ultrathin protoplast sections suggests that those structures may play an important role as carriers of enzymes of precursors of the wall by a process equivalent to reverse pynocytosis (Villanueva, 1966).

Dr C. Strunk (personal communication) informed us that reversion in protoplasts of *Polystictus versicolor*, obtained by the help of snail enzyme preparation, occurred under suitable conditions in protoplasts which contained two nuclei. Results similar to those described by us in relation to the way in which reversion is produced were also seen; the direct reversion to a hypha which was the beginning of normal mycelium was relatively rare. Most frequently abnormal bud-like forms arose as for the protoplasts of Fusarium and other fungi.

Prolonged incubation of the first generation of globular forms from *Fusarium culmorum* invariably resulted in mass reversion to the mycelial state. This occurred through an initial increase in the number of large bodies following their separation and in the formation of enlarged filamentous forms. It should be emphasized, however, that all these reversion studies were made on semisolid medium, which is a more effective means of achieving reversion than on solid or liquid media.

Effect of the physical state of the regeneration media

It has been stated by various workers that the physical state of the regeneration media is a very important factor affecting the phenomenon. Necas (1965) studied the regeneration of protoplasts of *Saccharomyces cerevisiae*, obtained by an autolytic process, and reported that protoplasts did not regenerate in liquid medium. On the other hand, nearly 100 % regeneration was achieved in 24 hr by cultivation in the same medium solidified by the addition of gelatin (15 %). Any other substance used to solidify the medium did not give satisfactory results. Regeneration did not take place on the surface of this solid medium, but only when the protoplasts were immersed inside of it. Our results with *Fusarium culmorum* protoplasts were quite different to those of Necas. Reversion of *F. culmorum* protoplasts to normal hyphae was readily obtained in semisolid agar (0·2 %, w/v), but reversion was also observed on incubation of protoplasts in solid (2 %, w/v) agar, in gelatin (0·2–20 %) and in liquid media (Table 1). Solid media allowed regeneration of *F. culmorum* protoplasts even on the surface. Under the optimal conditions for regeneration (2 % sucrose, 10 % sorbose) not more than 3 % regeneration was obtained using gelatin (0·2–20 %). Only on the surface of plates solidified with 10 % gelatin and seeded with protoplasts did we get a better rate of regeneration. On the other hand, use of 0·2 % agar, instead of gelatin, gave over 80 % regeneration.

The reversion to mycelial forms of *Fusarium culmorum* from spherical protoplasts was followed conveniently in liquid medium, using microdroplets of regeneration medium containing sucrose + sorbose. In this medium, the uniformity of external pressure, the elasticity of the newly formed membrane and interferencial tension, all tended to conserve the more or less spherical shape of the protoplast. The development of regenerated masses from single protoplasts during the initial stages were similar to the evolution in semisolid medium. A number of protuberances and growing spheres were also formed. At times there were seen local points of weakness where the growing protoplast pinched off a number of small blebs (Pl. 3, fig. 7) which sometimes gave ameboid forms. During the very first stages of regeneration the regenerated forms were osmotically fragile, but once the ameboid and elongated forms were formed the osmotic sensitivity partially disappeared.

Table 1. *Influence of the physical state of the media on regeneration*
of fusarium protoplasts

The basal medium contained the mineral salts of the Czapek medium in distilled water to which sucrose (2 %) and sorbose (10 %) were added

Basal medium plus	Protoplast regeneration after incubation for 17 hr (%)
None	65
Agar (0·2 %)	82
Agar (2 %)	52
Gelatin (0·2 %)	2
Gelatin (1·0 %)	3

Effect of modifications in the composition of the growth medium
on regeneration

Since *Fusarium culmorum* grows very well in Czapek medium, this was used as basal medium for the regeneration of protoplasts from mycelium. The best results were obtained with media containing the salts of the Czapek medium + 10 % (w/v) sorbose + 2 % (w/v) sucrose + 0·2 % (w/v) agar (Table 2). This medium was used throughout our work, unless otherwise stated. Concentrations of sorbose (Table 3) between 2 and 5 % produced little regeneration, and some lysis of the protoplasts. Concentrations of 10–12·5 % were optimal; higher concentrations gave somewhat lower degrees of regeneration. The sole difference found was the increasing refringency of the protoplast contents as the sorbose concentration was increased. No other sugar tested could replace sorbose, but sorbitol replaced sorbose.

Neither glucose nor fructose could replace the sucrose which was used in admixture with sorbose. Glucose + fructose satisfactorily replaced for sucrose. Increasing the concentrations of sucrose and decreasing those of sorbose gave somewhat lower degrees of regeneration (Table 4).

Since there was a possibility that impurities in the sorbose preparation used might affect protoplast regeneration, samples of sorbose from different sources (Merck, Sigma, Pfanstiehl) were tested; the results were very similar with all.

The influence of the nitrogen source of the regeneration media was examined by

replacing the NaNO₃ of the Czapek medium by different nitrogen compounds (NH_4NO_3, $(NH_4)_2SO_4$, glutamate, glycine, asparagin, casein, peptone). Casein was the best nitrogen source, but no great difference was observed of the other organic and inorganic N sources.

Table 2. *Effect of various carbon sources on the regeneration of*
Fusarium culmorum protoplasts

The basal medium was formed by the mineral salts of Czapek medium in distilled water + 0·2 % agar. All concentrations are expressed in w/v.

Basal medium plus	Protoplast regeneration after incubation for 17 hr (%)
Xylose (10 %)	10
Glucose (10 %)	12
Fructose (10 %)	11
Galactose (10 %)	24
Mannose (10 %)	14
Sorbose (10 %)	6
Sucrose (10 %)	11
Sucrose (2 %) + mannitol (14·5 %)	28
Sucrose (2 %) + xylose (10 %)	5
Sucrose (2 %) + sorbose (10 %)	78
Sucrose (10 %) + sorbose (2 %)	13
Sorbose (2 %) + xylose (10 %)	8
Sorbose (2 %) + xylose (2 %)	12 (lysis)

Table 3. *Effect of sucrose and increasing amounts of sorbose on the*
regeneration of Fusarium culmorum protoplasts

To the basal medium previously described the various concentrations of sugars were added

Basal medium plus		Protoplast regeneration after incubation for 17 hr (%)
Sucrose (%)	Sorbose (%)	
2	2	12 (lysis)
2	5	25 (some lysis)
2	10	78
2	12, 5	82
2	15	70
2	17, 5	74
2	20	68

Effect of environmental conditions on the capacity for regeneration

The influence of several environmental and chemical factors on regeneration from protoplasts of *Fusarium culmorum* was examined. Under the optimal conditions previously described, temperatures between 18° and 33° did not significantly affect the number of protoplasts able to regenerate in semisolid or liquid medium. When incubation was at 28° in liquid media aeration had no noticeable effect; shaken and static cultures showed about the same degree of regeneration.

Different pH values of the medium showed marked effects on regeneration. When protoplasts were incubated at 28° statically the maximum regeneration (70 %) was at about pH 6·7. At pH 5, 50 % of the protoplasts regenerated; at pH 8 only 12 % of the protoplasts reverted to normal mycelium. At pH 9–10 regeneration did not take place; at pH 3, 25 % of the protoplasts regenerated. At greater than pH 8, regeneration did not occur under our conditions. The curve relating regeneration and pH value was linear from pH 3 to 6·5, and decreased sharply above pH 7.

Table 4. *Effect of various concentrations of sucrose (or glucose + fructose) and sorbose on the regeneration of Fusarium culmorum protoplasts*

To the basal medium previously described the various concentrations of sugars were added

Basal medium plus	Protoplast regeneration after incubation for 17 hr (%)
% %	
Sucrose (2) + sorbose (10)	75
Sucrose (4) + sorbose (8)	56
Sucrose (6) + sorbose (6)	27
Sucrose (8) + sorbose (4)	15
Sucrose (10) + sorbose (2)	11
Glucose (2) + sorbose (10)	13
Fructose (2) + sorbose (10)	11
Glucose (1) + fructose (1) + sorbose (10 %)	80

Attempts to prevent protoplast reversion

Attempts to prevent reversion from protoplast to the mycelial form were made by subculturing the initial globular forms in a variety of media. We were not able to show the presence of permanently stable globular forms, possibly because of some factors which affect the successive generations, with a resulting increase in appearance of filamentous forms. The globular proliferation showed intermediate forms more transparent and with less consistency. When protoplasts were kept for 15–20 days in the presence of the lytic enzyme as during protoplast formation, the spherical bodies did not regenerate but many remained alive, as indicated by the vital staining technique, and were able to regenerate when transferred to new media.

Regeneration in other fusarium species

Reversion from protoplast to mycelial form was noted with all the *Fusarium* species which we tested (*F. oxysporium, F. avenaceum, F. cubense, F. solani, F. roseum, F. poae, F. decemcellulare, F. orthrosporioides*), the intermediate stages of the process in general being very like to that described for *F. culmorum*. However, with *F. orthoceras* very abnormal mycelium was regenerated.

Comparison of the regeneration process with protoplasts obtained by means of snail and other lytic enzyme preparation

In the previous experiments protoplasts were always obtained by cell-wall digestion with strepzyme RA (Rodriguez Aguirre *et al.* 1964). Some experiments

were done by digestion of *Fusarium culmorum* mycelium with the digestive juice of *Helix pomatia* under the same experimental conditions as used with strepzyme RA. Apart from the morphological similarity of protoplasts obtained with strepzyme RA and snail enzyme, perhaps the most direct evidence of similarity was the analysis of the stabilized protoplasts which did not show any residues of materials corresponding to cell walls.

Regeneration of the protoplasts obtained by means of the snail enzyme preparation follows steps very similar, nearly identical, to those described for protoplasts obtained with strepzyme RA. The only significant difference was the formation of a large number of convoluted forms in the protoplasts obtained by strepzyme RA. Regeneration studies with protoplasts obtained with snail enzyme preparations were often complicated by bacterial contamination. Attempts made to obtain protoplasts of *Fusarium culmorum* by growing the organism on a medium containing high concentrations of sorbose, according to Hamilton & Calvet (1964), were unsuccessful.

The results here suggest that regeneration of protoplasts to the mycelial form and cell wall formation *de novo* in *Fusarium culmorum* can take place and that a very high proportion retain the ability to regenerate and to form new normal cells. However, the process is a slow one. Whether the low rate of regeneration found in these experiments was due to unsatisfactory cultural conditions or to the lack of nuclei in some of them is not yet known.

REFERENCES

BACHMANN, B. J. & BONNER, D. M. (1959). Protoplasts from *Neurospora crassa*. *J. Bact.* **78**, 550.

EMERSON, S. & EMERSON, M. R. (1958). Production and reversion of protoplast-like structures in the osmotic strain of *Neurospora crassa*. *Proc. natn. Acad. Sci. U.S.A.* **44**, 668.

GANCEVICI, G., STOIAN, I. & KELLER, Z. (1962). Localisation cytochimique des graisses dans les cultures de tissus. *Archs. roum. Path. exp. Microbiol.* **21**, 191.

GARCÍA ACHA, I., LÓPEZ-BELMONTE, F. & VILLANUEVA, J. R. (1966). Obtention of protoplasts from fungal spores. *Antonie van Leeuwenhoek* **32**, 299.

HAMILTON, J. G. & CALVET, J. (1964). Production of protoplasts in an osmotic mutant of *Neurospora crassa* without added enzyme. *J. Bact.* **88**, 1084.

LÓPEZ-BELMONTE, F., GARCÍA ACHA, I. & VILLANUEVA, J. R. (1966). Observations on protoplasts of *Fusorium culmorum* and on their fusion. *J. gen. Microbiol.* **45**, 127.

NECAS, O. (1965). Mechanisms of regeneration of yeast protoplasts. II. Formation of the cell wall de novo. *Folia biol., Praha* **11**, 97.

RODRIGUEZ AGUIRRE, M. J., GARCÍA ACHA, I. & VILLANUEVA, J. R. (1964). Formation of protoplasts of *F. culmorum* by strepzyme. *Antonie van Leeuwenhoek* **30**, 33.

ROST, K. & VENNER, H. (1965). Wachstum von Hefeprotoplasten und ihre Reversion zu intakten Hefezellen. *Archiv Mikrobiol.* **51**, 130.

STRUNK, Ch. (1965). Entstehung und Reversion von Basidiomyceten-Protoplasten. In *Symposium über Hefe-Protoplasten*, Ed. by R. Müller Berlin: Akademie Verlag.

VILLANUEVA, J. R. (1966). Protoplasts of Fungi. In *The Fungi*. Ed. by G. C. Ainsworth & A. S. Sussman. Vol. 2, p. 2. New York: Academic Press.

I. GARCÍA ACHA AND OTHERS

I. GARCÍA ACHA AND OTHERS
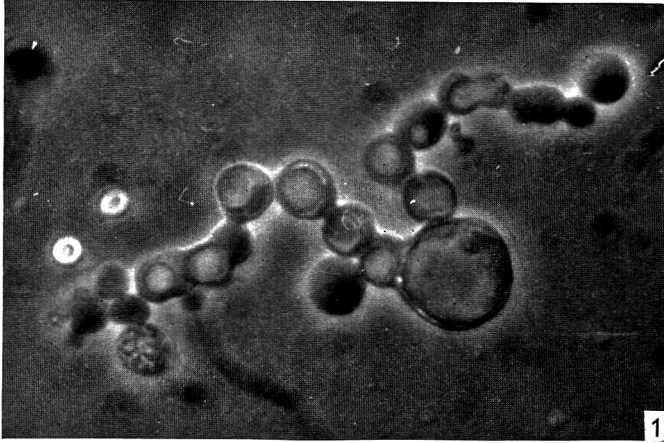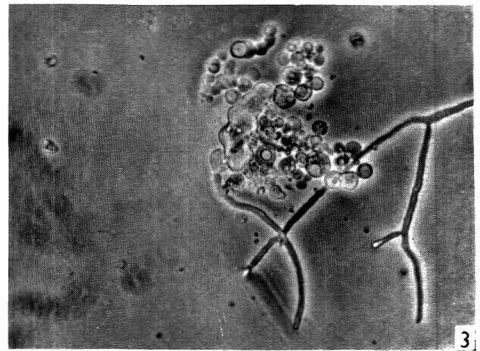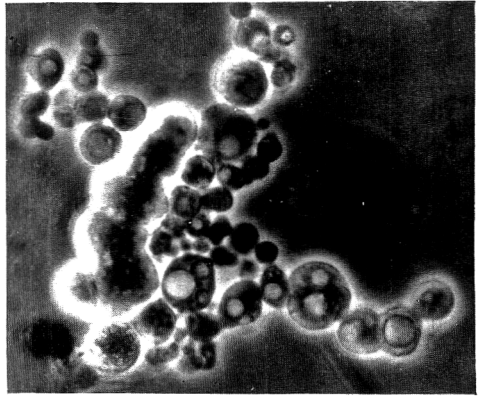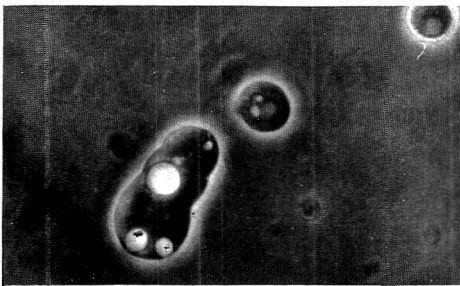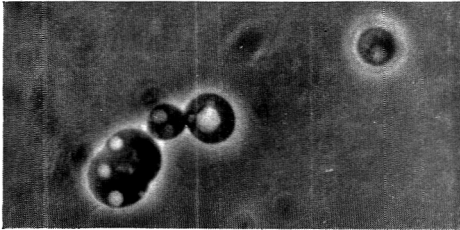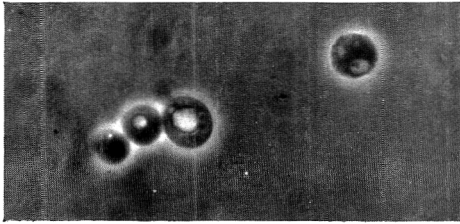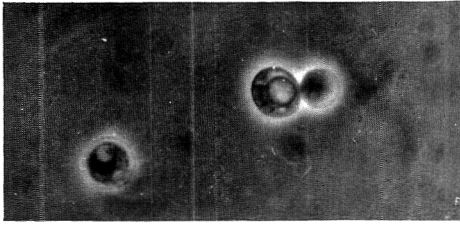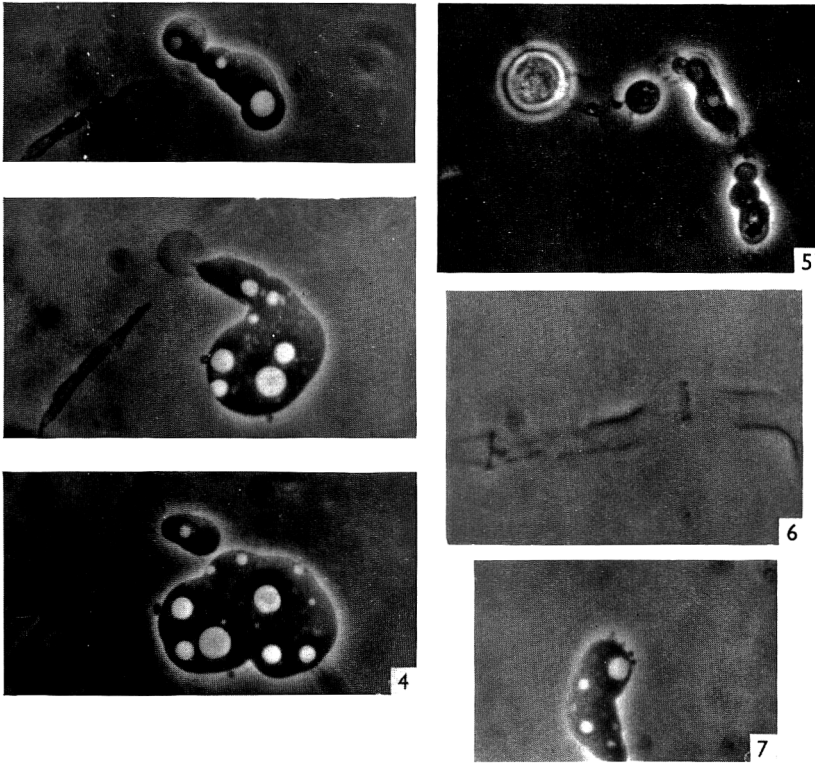
EXPLANATION OF PLATES

PLATE 1

Fig. 1. Regeneration of *Fusarium culmorum* protoplasts. Note the series of yeast-like forms (× 6000).

Fig. 2. Direct formation of a hypha from a *F. culmorum* protoplast (× 6000).

PLATE 2

Fig. 3. Another type of regeneration in *F. culmorum* protoplast. Note the formation of large ameboid forms (× 6000).

PLATE 3

Fig. 4. Emigration of the protoplast contain showing the remaining empty shells (× 6000).

Fig. 5. Another example of shell liberation during the regeneration process in *F. culmorum* protoplasts (× 6000).

Fig. 6. Empty cell walls of *F. culmorum* remaining in the preparation once that the protoplasts were released (× 6000).

Fig. 7. Ameboid form of a regenerating protoplast of *F. culmorum* showing the formation of small blebs (× 6000).

Note added in proof

Trehalose has been used instead of sucrose in combination with sorbose for the process of regeneration of *Fusarium culmorum* protoplasts but although regeneration occurs it works very slowly: after 4 days incubation normal hyphae are not yet formed, and the chains of regenerated cellular aggregates are very short.

Fragmentation in *Nocardia corallina*

By O. BROWN* and J. B. CLARK

Department of Botany and Microbiology, University of Oklahoma,
Norman, Oklahoma, U.S.A.

SUMMARY

The growth cycle of *Nocardia corallina* involves a period of coenocytic hyphal development terminated by fragmentation. Hyphal elongation occurred by insertion of new material at the hyphal tips, not by intercalary growth. Exceptions occurred at points of branch initiation. Development of cross-walls preceded fragmentation and the new tips so produced were also capable of elongation. The fragmentation process appeared to be initiated by agents which accumulated in the medium, since used broth from actively fragmenting cultures stimulated earlier fragmentation in assay inocula. This effect was not shown by media from cultures in the coenocytic hyphal phase, nor by old resting cultures. The population density also affected fragmentation in a manner which suggested that accumulation of threshold concentrations of a diffusible metabolic product was required. A Millipore filter technique for assaying the effects of agents involved in fragmentation is described.

INTRODUCTION

Members of the genus *Nocardia* have similar growth cycles which involve periods of hyphal development terminated by fragmentation. The extent and duration of hyphal development vary with environmental conditions, but have been used as criteria for delineation of the genus as well as for speciation (McClung, 1949, 1954). The growth cycles of several species have been carefully described, but the mechanisms which control morphogenesis are unknown. However, fragmentation in *Nocardia corallina* appears to be induced by agents which accumulate in the growth medium (Webb & Clark, 1957). A further study of this phenomenon is reported here, dealing specifically with the fragmentation sequence, additional evidence for metabolic factors involved in fragmentation, and a method of assaying such factors.

METHODS

Organism. Nocardia corallina ATCC 4273 was maintained on nutrient agar + 0·5 % fructose. Cultures were transferred at 48-hr intervals (a time sufficient for completion of the growth cycle) to provide reproducible inocula for all experiments.

Observation of growing cultures. Agar block cultures were grown at 25°–26° under coverslips on hanging-drop slides by using procedures similar to those described by McClung (1949). The agar blocks were cut from nutrient agar containing 0·5 % glucose, and the coverslips were sealed only on two sides and positioned off-centre

* Present address: Department of Microbiology, School of Medicine, University of Missouri, Columbia, Missouri. U.S.A.

to provide an opening for diffusion of air into the chamber. The development of individual organisms was microscopically observed and photographed at intervals.

Photomicrography. Photomicrographs were taken on Kodak High Contrast 35 mm. film with an apochromatic lens system (limiting n.a., 1·3). Living organisms were photographed with the substage condenser diaphragm almost closed to give maximum contrast and to expose the organisms to a minimum of light. Phase microscopy was attempted, but a higher light intensity was then required and the organisms did not germinate normally.

Fragmentation studies. Growth on solid medium was measured by using a series of Millipore filters inoculated with equal numbers of organisms and incubated at 29° on the surface of semi-solid nutrient agar (0·75 % agar) containing 0·5 % fructose. The Millipore filters were removed after various intervals of incubation and placed in screw-cap tubes containing 0·9 % NaCl solution and large beads. The organisms were freed from the filter and evenly suspended by mixing first with the Vortex Jr. mixer (Scientific Industries, Inc., Queens Village, N.Y.) and then with the Disontegrator (Ultrasonic Industries, Inc., Albertson, L.I., N.Y.). Viable units were determined by standard plate colony-counting techniques. Slides were made at intervals and stained by the Chance nuclear stain (Chance, 1952) or the Webb cell-wall stain (Webb, 1954).

Detection of fragmentation stimulation. Cultures were grown in nutrient broth + 0·5 % fructose and aerated with sterile air under pressure. Samples of culture were removed after various intervals of incubation and freed from organisms by centrifugation and filtration; culture filtrates so prepared will hereafter be referred to as 'used media'. Preliminary assays were done by inoculating the used media as described by Webb & Clark (1957). Several advantages were found for the following modification, which uses surface cultures rather than broth cultures for assay. The assay inoculum was prepared as follows. The growth from 48-hr cultures was harvested from fructose agar and washed three times with 0·9 % NaCl solution by alternate suspension and centrifugation for 10 min. at 10,000g in a Servall refrigerated centrifuge at 4°. A standard curve, relating extinction and number of colony-forming units, was prepared for convenience in reproducing inoculum sizes. Equal samples were filtered through Millipore filters (HAWP 0·45 μ), thus producing a series of filters inoculated with equal numbers of evenly distributed organisms. The filters were transferred to sterile Millipore pads, saturated with fresh fructose broth for control cultures, or with samples of used medium for determining fragmentation-stimulation activity. After intervals of incubation, slides were prepared and evaluated as previously described. An inoculum of 10^7 organisms/filter with evaluation of slides after 6 hr was found to be adequate for the assay.

RESULTS

Fragmentation

The fragmentation sequence of a developing microcolony is shown in Plates 1–3, figs. 1–13. Routinely, the initial cross-wall formed near the centre of the hypha (fig. 1). After fragmentation, elongation occurred from both the new tips. Comparisons of Pl. 1, figs. 1–3, show that elongation occurred only at the tips of the hyphae, including the new tips produced by fragmentation. Intercalary growth did not occur, except at

the point of branch initiation (Pl. 1, fig. 4). This is more clearly shown in Fig. 1 which depicts results of measurements made with a filar micrometer on developing hyphae with multiple branches which served as markers. The average rate of elongation for the two ends of the original filament was the same, about 0·02 μ/min. The average rate of increase for the newly developing branches was slightly less.
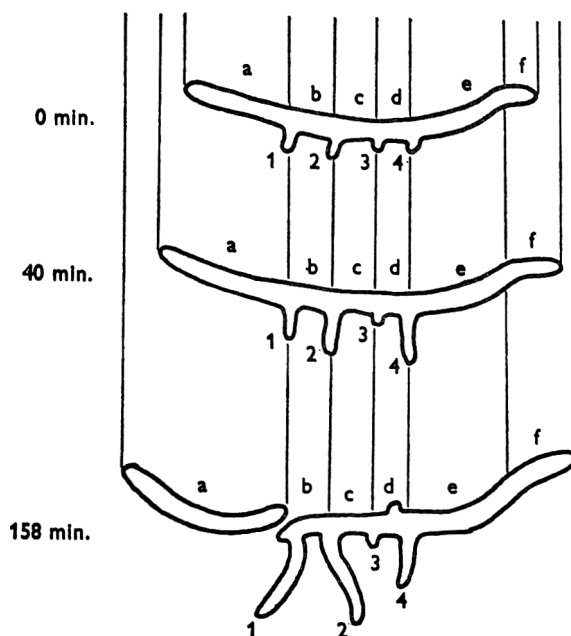
Fig. 1. Growth of hyphae of *Nocardia corallina* by elongation from the tips. The values listed below give the growth rates for the indicated portions of the hyphae shown in the figure:

Hyphal segment (letter) or branch (number)	Length (μ) at			Total change in length (μ)
	0 min.	40 min.	158 min.	
a	5·34	6·10	8·45	+3·11
b	2·12	2·07	2·09	−0·03
c	1·91	2·38	2·02	+0·11
d	1·69	1·45	1·48	−0·21
e	3·39	3·49	3·37	−0·02
f	1·57	2·03	4·67	+3·10
1	1·67	2·16	4·30	+2·63
2	1·73	2·50	4·52	+2·79
3	0·57	0·71	0·57	0·00
4	0·89	1·01	2·92	+2·03

In Pl. 1, fig. 5, and Pl. 2, fig. 6, the terminal portions of the original hyphae are still coenocytic and elongating. In Plate-figs. 7–10 (Pls. 2, 3), a wave of cross-wall formation has progressed outward from the middle, producing short fragments of approximately equal length. Elongation had essentially ceased in figs. 11–13 (Pl. 3), but cross-walls continued to be formed and a uniform population of short fragments was produced; in fig. 13 some coccoid elements are to be seen.

Fragmentation stimulation by cell-free culture filtrates

Fragmentation was not stimulated by medium from 1-day cultures nor by medium from week-old growth. Used media from 2-, 3- and 4-day cultures showed progressively greater fragmentation-stimulation of assay inocula. Cytologically normal germination occurred in all cases, but the coenocytic hyphal phase was shortened because of earlier and more extensive formation of cross-walls. With 3- and 4-day used media, cross-wall formation began after only a few nuclear divisions, while the hyphae were still very short. The typical development of long coenocytic hyphae as seen in control cultures was not observed. The fragmentation stimulation effects of used media were not destroyed by boiling for 5 min.

Figure 14 depicts typical results obtained by the Millipore filter assay technique. The control (Fig. 14A) supplied with fresh nutrient broth is typical of a 6 hr culture under these conditions. Fig. 14B shows a culture grown for the same period in used media taken from a 3-day fragmenting culture. Most of the organisms contain multiple cross-walls and some are fragmenting.

Table 1. *Effects of inoculum size upon fragmentation in cultures of*
Nocardia corallina grown on the surface of Millipore filters

Total no. organisms inoculated/filter	Organisms inoculated/ μ^2 surface area of medium	Onset of fragmentation (hr)
4×10^5	0·0042	22
4×10^6	0·042	14
4×10^7	0·42	12–14
4×10^8	4·2	No coenocytic hyphal phase; initial growth with cross-wall formation

Table 2. *Growth rates of Nocardia corallina on Millipore filters*

Incubation time (hr)	Extinction at 475 mμ*	Organisms (colony count)/filter ($\times 10^{-8}$)
0†	0·035	4·3
0	—	4·1
5·5	0·128	6·5
8·25	0·205	13·8
8·8	0·207	20·9
20·5	0·581	54·0
32·5	0·964	91·6

* Total filter population resuspended in 10 ml. of 0·9 % NaCl solution.

† Figures in this row were obtained from a sample of the suspension subsequently used to inoculate the filters.

Effects of population density upon fragmentation

The duration of the coenocytic hyphal phase was also dependent upon the initial inoculum size; with larger inocula, fragmentation occurred sooner (Table 1). When the inoculum was increased to give more than a monolayer, e.g. 4·2 organisms/μ^2 surface, the typical coenocytic phase did not occur. Cytological evidence indicated that such organisms were growing by elongation and multiplying by cross-wall pro-

duction at, essentially, each nuclear division. To gain further evidence about multiplication under these conditions, the growth rates of surface colonies were determined (Table 2). The inoculum, $4\cdot3 \times 10^8$ organisms/Millipore filter, was sufficient to provide a layer several organisms thick. Under these conditions, proliferation did occur, but the normal phase of long coenocytic hyphae (as shown in Plate 1, fig. 1) did not occur. Instead, cross-walls were produced soon after germination and the culture had an appearance similar to that shown in Plate 4, fig. 14 B.

The data in Table 2 also provide information about the increase in cell mass relative to proliferation by fragmentation. The extinction increased by a factor of $3\cdot6$ during the first $5\cdot5$ hr, while the number of organisms (colony count) increased by $1\cdot5$. This indicated an increase in size of organism and some multiplication, in agreement with the cytological observations. During the period, $5\cdot5$–$8\cdot25$ hr, the extinction increased by a factor of $1\cdot6$ while the population (colony count) doubled. This also agreed with cytological evidence that elongation was accompanied by fragmentation. During the period, $8\cdot25$–$8\cdot8$ hr, there was essentially no increase in extinction; however, there was an increase of $1\cdot5$ in numbers of organisms (colony counts), which indicated that some fragmentation occurred. During the period, $8\cdot8$–$20\cdot5$ hr, the increases in extinction and in population were $2\cdot8$ and $2\cdot6$, respectively, indicating that fragmentation kept pace with synthesis of new cell mass. This consistent relationship continued through the $32\cdot5$ hr observation with ratios near $1\cdot7$. For the total $32\cdot5$ hr period, the extinction increased by a factor of $27\cdot5$ while the population increased by a factor of $22\cdot3$.

DISCUSSION

Observations of growing *Nocardia corallina* indicated that insertion of new cell-wall material, which accompanied growth and elongation, occurred at the tips but not along interior portions of hyphae. Exceptions occurred wherever cross-walls formed; the two new tips, produced then, served as loci for elongation. In addition, the origin of branches represented loci where new cell-wall material was inserted. At present it is not clear whether these three germinal areas share common mechanisms.

Since intercalary growth did not occur, and cross-septation initially occurred near the middle of a given hypha and progressed down the filament toward the tip, it follows that fragmentation occurred first in the older parts of the hypha. Since fragmentation resulted in production of more growing tips, there was a greater proliferation near the origin of a developing microcolony. It seems reasonable that this region is also the area where diffusible metabolic products would accumulate most rapidly. During the early stages of growth, the elongating tips, which remain free from cross-walls for the longest period, are being extended into a fresh environment and the pattern of fragmentation seen in figs. 1–13, Pls. 1–3, is consistent with what would be predicted if fragmentation were induced by a diffusible metabolic product.

The fragmentation-stimulation effect of used medium was correlated with growth cycle stages which actively formed cross-walls. Its absence from young cultures in coenocytic growth and week-old non-multiplying cultures suggests that the agent responsible is produced by the developing culture and subsequently used by the organisms or is decomposed because of inherent lability.

Germination to coenocytic hyphae is typically associated with the process of

34

transfer which initiates a new culture. Among other changes which occur during such transfers, there is a great decrease in the number of organisms. If fragmentation were dependent upon some critical concentration of metabolic by-products, then transfer would remove the organisms from such products and the decrease in population would decrease their rate of re-accumulation. The coenocytic phase would then coincide with the time required for the fragmentation factors to accumulate to threshold concentration. If such were the case, the effects of culture transfer to new medium should be partially negated by transferring a mass inoculum, since a high population density should result in a more rapid accumulation of the metabolic factors involved. Tables 1 and 2 show that such an effect was obtained with mass inocula which were washed to remove most of the extracellular metabolic by-products. When these cultures were initiated with inocula containing sufficient organisms to cover the surface completely, the typical coenocytic hyphal phase did not occur and growth and multiplication occurred by cytokinesis accompanied by karyokinesis. This same type of growth and multiplication is typical of normal cultures only after the population has developed to a similar density through germination, coenocytic hyphal development and stages of accelerated cross-wall formation.

The Millipore filter assay technique offers several advantages for studying the fragmentation-stimulation effects of used media. The process is rapid, requiring only 6 hr incubation, and the development of the growth cycle in control cultures is uniform, with fragmentation a more synchronous and more sharply defined event than that seen in broth cultures; hence the effects of used media are more clearly shown. The filter technique also allows accurate reproduction of population density and distribution. With the availability of this assay procedure it appears that further study of metabolic factors involved in morphogenesis of the nocardias will be feasible.

REFERENCES

CHANCE, H. L. (1952). Crystal violet as a nuclear stain for *Gaffkya tetragena* and other bacteria. *Stain Technol.* **27**, 253.

McCLUNG, N. M. (1949). Morphological studies in the genus *Nocardia*. I. Developmental studies. *Lloydia* **12**, 137.

McCLUNG, N. M. (1954). Morphological studies in the genus *Nocardia*. III. The morphology of young cultures. *Ann. N.Y. Acad. Sci.* **60**, 169.

WEBB, R. B. (1954). A useful bacterial cell wall stain. *J. Bact.* **67**, 252.

WEBB, R. B. & CLARK, J. B. (1957). Cytogenetic study of *Nocardia corallina. J. Bact.* **74**, 31.

EXPLANATION OF PLATES

PLATES 1–3

Figs. 1–13. The fragmentation sequence in *Nocardia corallina*. Photographs were taken at the following time intervals: fig. 1, 12 hr; fig. 2, 13 hr, 50 min.; fig. 3, 15 hr, 15 min.; fig. 4, 16 hr, 55 min.; fig. 5, 18 hr, 30 min.; fig. 6, 20 hr, 45 min.; fig. 7, 24 hr, 45 min.; fig. 8, 27 hr, 10 min.; fig. 9, 28 hr, 10 min.; fig. 10, 30 hr, 40 min.; fig. 11, 32 hr, 40 min.; fig. 12, 34 hr, 40 min.; fig. 13, 39 hr, 40 min. × 3500.

PLATE 4

Fig. 14. Stimulation of cross-wall formation by used medium. The control culture of *Nocardia corallina* (A) was grown on a Millipore filter placed on a pad saturated with fresh nutrient broth + 0·5 % fructose. The test culture (B) was treated identically except that used broth from a 3-day culture was substituted as nutrient. Both filters were inoculated by filtration with 10^7 organisms from the same suspension. Organisms were stained by the Webb cell-wall procedure (1954) and photographs were taken after incubation for 6 hr at 29°. × 3000.

O. BROWN AND J. B. CLARK

Plate 3

10

11

12

13

O. BROWN AND J. B. CLARK

Plate 4

14 A

14 B

O. BROWN AND J. B. CLARK

531
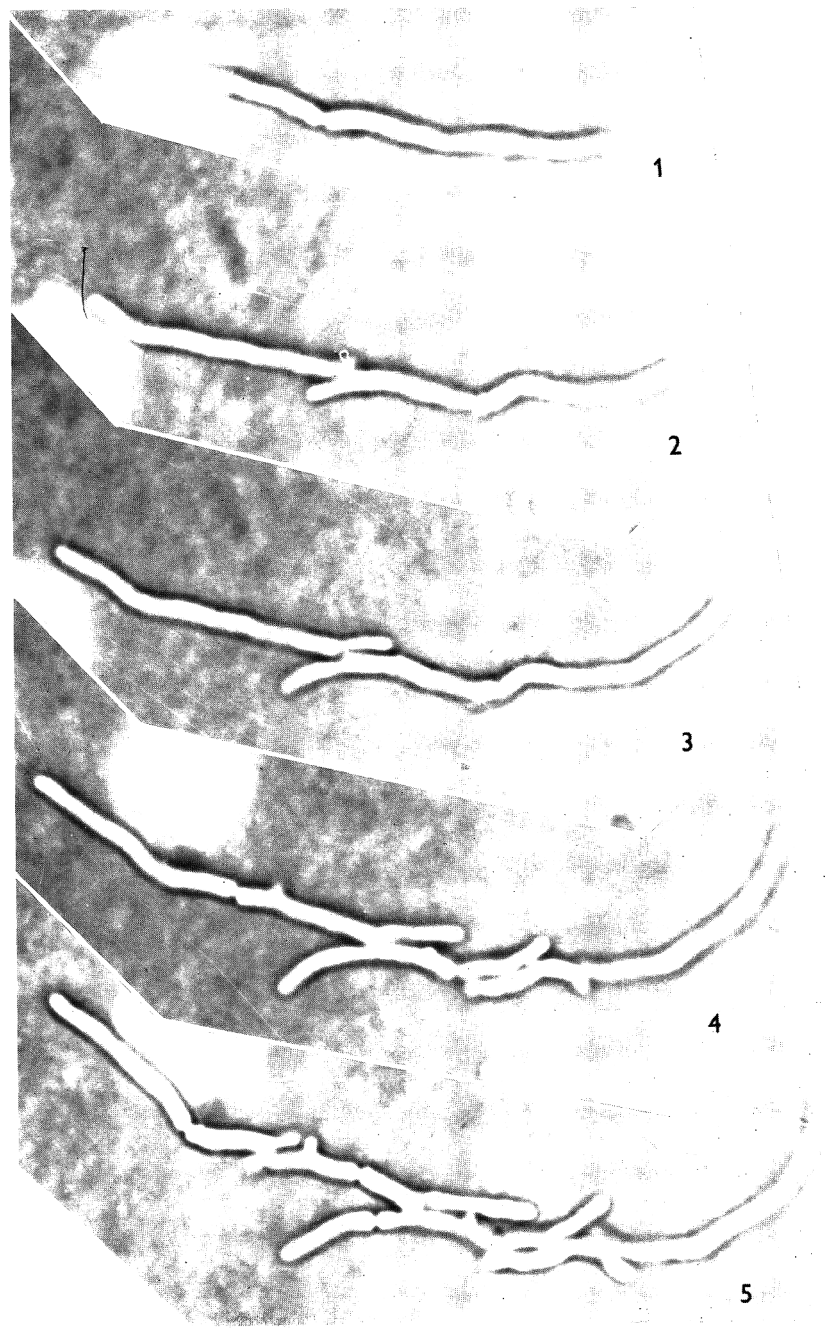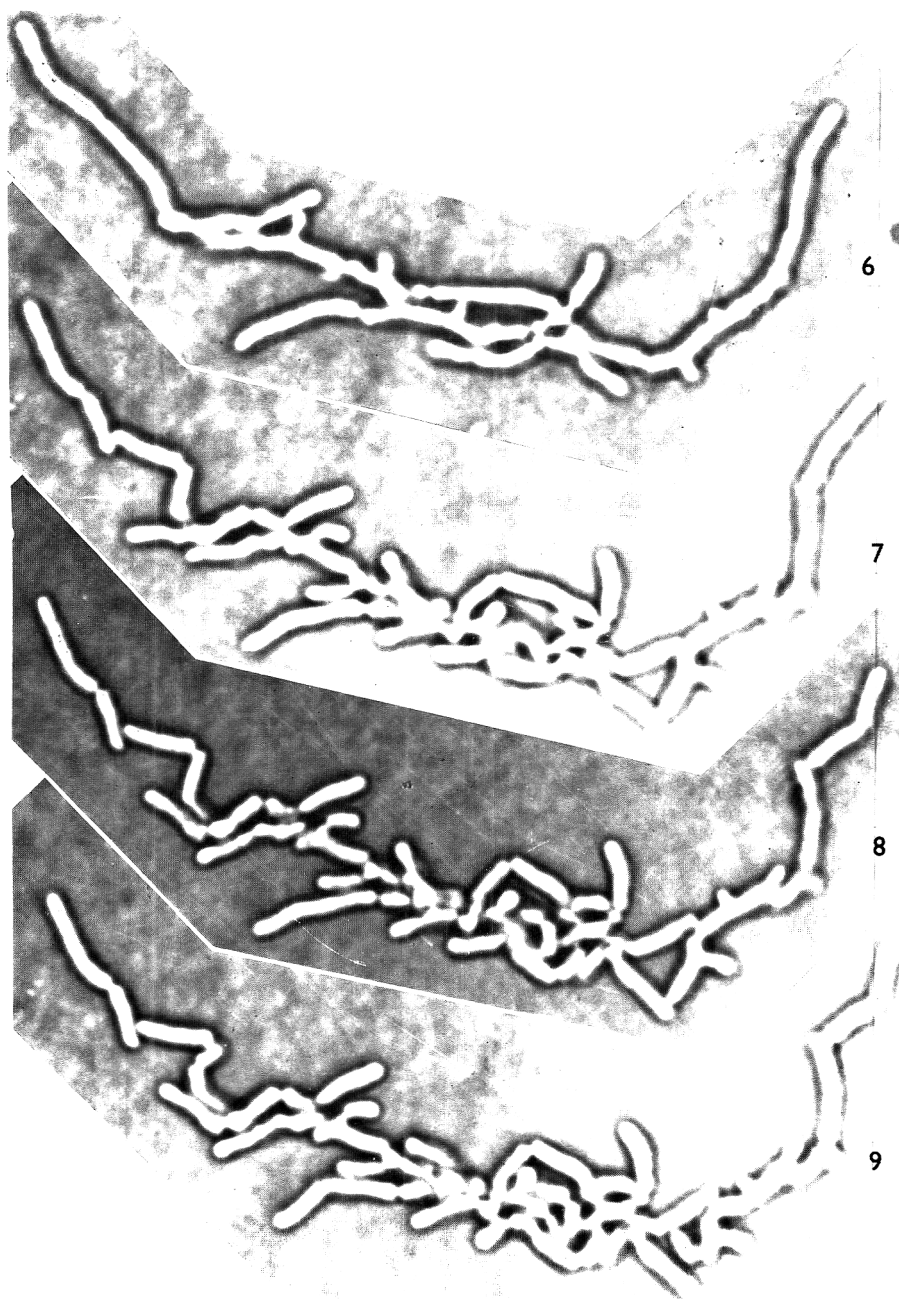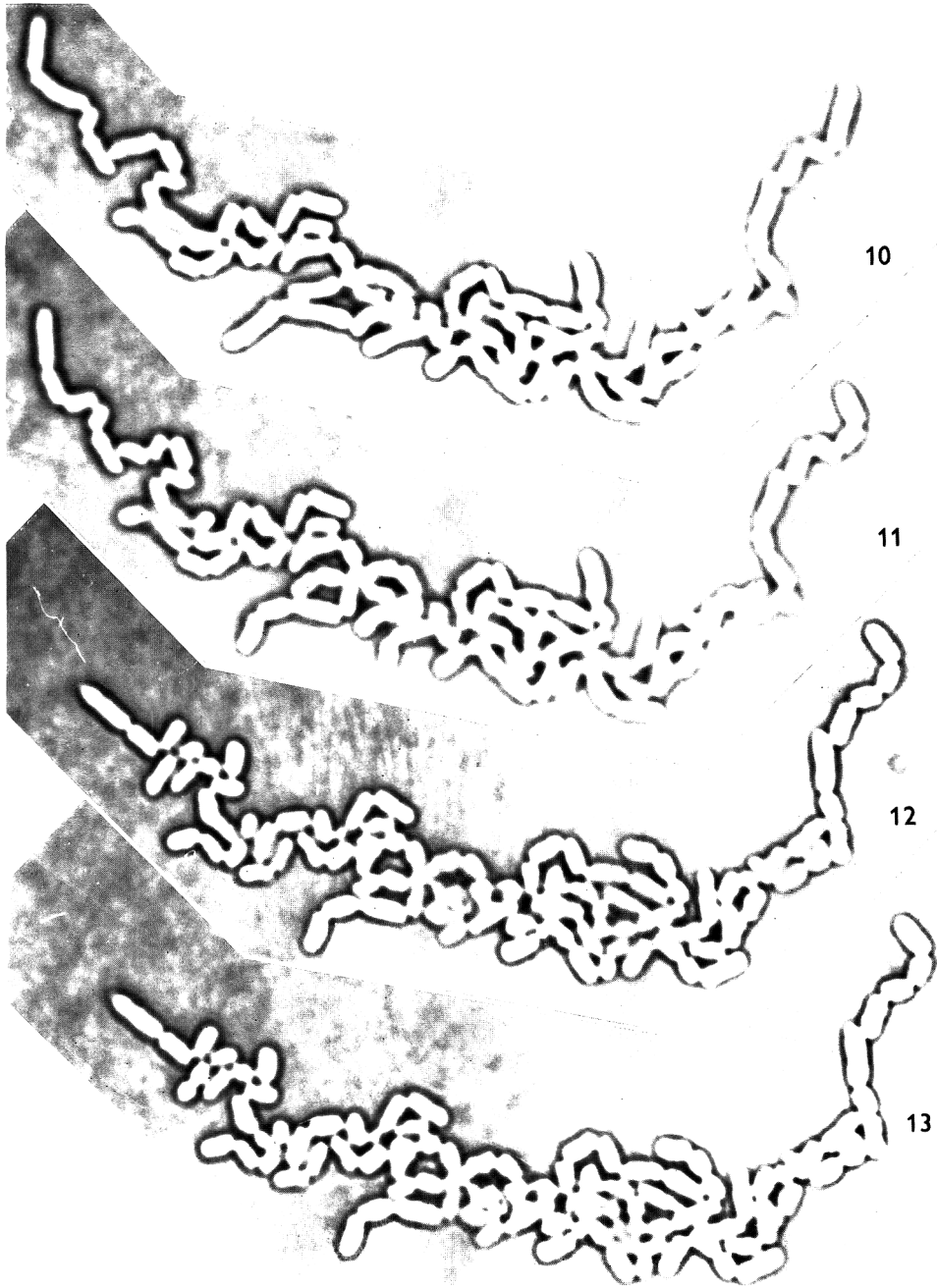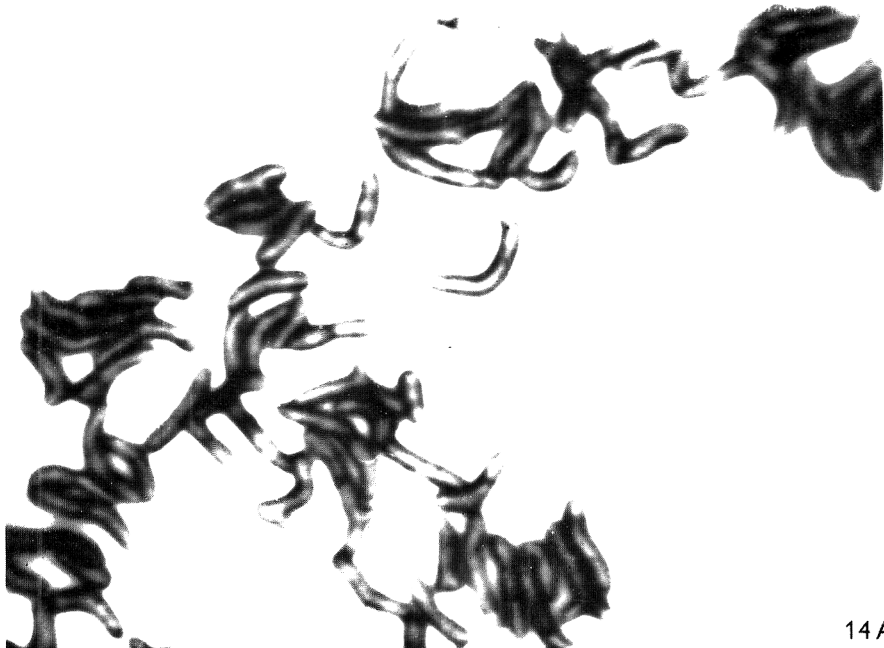
Inhibition of Carotenoid Synthesis in a Mutant
of *Verticillium albo-atrum*

By L. R. G. VALADON and ROSEMARY S. MUMMERY

*Department of Botany, Royal Holloway College (University of London),
Englefield Green, Surrey*

SUMMARY

Verticillium albo-atrum mutant M 5·6 which produces phytoene, β- and γ-carotene, neolycopene A, lycopene and neurosporaxanthin was subjected to the effect of the following inhibitors of carotenoid synthesis: diphenylamine, 2-hydroxydiphenyl, β-ionone, methylheptenone. Certain intermediates in the Porter–Anderson (1962) pathway for carotenoid biosynthesis not previously present were then observed, namely: phytofluene, β-zeacarotene, ζ-carotene, neurosporene. When the mutant was grown in the presence of diphenylamine, washed free from it and then grown again in 1 % glucose in M/15 KH_2PO_4, the more unsaturated polyenes seem to be formed at the expense of the more saturated ones. There was also evidence that γ-carotene was formed via β-zeacarotene and the sequence of carotenoid formation seems to agree very closely to that suggested by Porter & Anderson (1962).

INTRODUCTION

As a result of a study on various tomato crosses Porter & Lincoln (1950) proposed a stepwise interconversion of C_{40} polyenes as a mechanism of carotenoid biosynthesis, at a time when the exact structures of the intermediates were not known. Porter & Anderson (1962), with more information available, showed that the original scheme was to a large extent substantiated but had to be revised. The proposed pathway (Fig. 1) suggested that phytoene was dehydrogenated to phytofluene, to ζ-carotene, then to neurosporene.
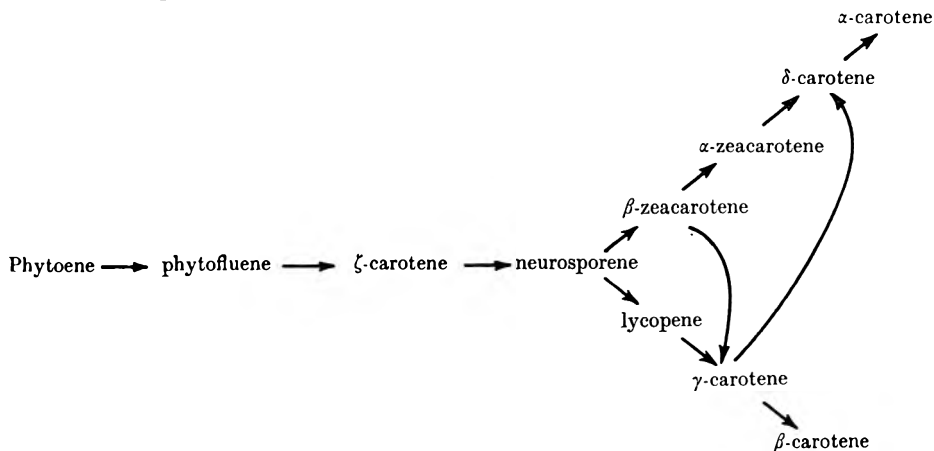
Fig. 1. Pathway of carotenoid biosynthesis suggested by Porter & Anderson (1962).

The cyclic β-carotene could be formed by cyclization of neurosporene to β-zea-carotene and also by cyclization of lycopene. There seems to be evidence that in fungi, it is neurosporene that is cyclized, and not lycopene: the intermediate β-zeacarotene has been identified in *Phycomyces blakesleeanus* (Davies, Villoutreix, Williams & Goodwin, 1963) and in *Rhodotorula glutinis* (Simpson, Nakayama & Chichester, 1964) and it was suggested in both of these that the main pathway between neurosporene and γ-carotene was mediated through β-zeacarotene. The structure of β-zeacarotene as determined by Rüegg *et al.* may support this view. Davies (1961) who worked on young and older cultures of *Rhizophlyctis rosea* with [^{14}C] labelled mevalonic acid obtained evidence that γ-carotene was not synthesized from lycopene.

An alternative to the Porter–Lincoln hypothesis was proposed by Goodwin (1952). He suggested that the series of polyenes could be produced independently from a common precursor; this is known as the independent hypothesis. Diphenylamine (DPA) inhibits carotenogenesis and when the colourless cultures of *Phycomyces blakesleeanus* were resuspended in DPA-free medium, it appeared that β-carotene was not being formed at the expense of the phytoene series (Goodwin, Jamikorn & Willmer, 1953). Results obtained by Zalokar (1954) with *Neurospora crassa* under different conditions of light and oxygen also support this hypothesis, since ζ-carotene, neurosporene and lycopene all appeared simultaneously. Krzeminski & Quackenbush (1960) by using the incorporation of mevalonic acid-2-^{14}C into the carotenoids of *N. crassa* showed clearly that the product/precursor relationship between the most unsaturated polyenes and the more saturated ones was not fulfilled. Further, results obtained by Villoutreix (1960) on ultraviolet mutants of *Rhodotorula mucilaginosa* and also of the effect of 2-hydroxydiphenyl on carotenogenesis support the independent hypothesis. Later work by Goodwin and co-workers (Davies *et al.* 1963) suggested that β-zeacarotene, phytofluene, ζ-carotene and neurosporene were formed from phytoene in the presence of DPA, and this is evidence for carotenoid synthesis along the Porter–Anderson (1962) pathway.

Results obtained by Valadon & Heale (1965) on mutants of *Verticillium albo-atrum* in which similar amounts of phytoene were obtained in the wild type (which did not contain carotenoids) and in five mutants (which contained carotenoids) did not seem to support the first hypothesis. However, it is known that precursor/product relationships are not always reflected in their quantities, or even in the detectability of the compounds involved (Dr D. G. Anderson, personal communication) and, as Valadon & Heale (1965) suggested, conclusions based on that type of evidence must be tentative. Inhibitors of carotenoid synthesis are well known, namely diphenyl-amine (Goodwin, 1952; Turian, 1957; Turian & Haxo, 1954) methylheptenone (Chichester, Wong & Mackinney, 1954; Nakayama, Chichester, Lukton & Mackinney, 1957) β-ionone (Mackinney, Nakayama, Buss & Chichester 1952); 2-hydroxy-diphenyl (Villoutreix, 1960). Usually after treatment with these inhibitors previously unobserved carotenoids are obtained, indicating that pathways for their formation are available in the given organism (Simpson *et al.* 1964). This prompted further work with *V. albo-atrum* with the hope of elucidating the synthesis of carotenoids in this organism.

METHODS

The culture of *Verticillium albo-atrum* Reinke & Berthold used in this work is designated M5·6. It is a subculture of the original M5 orange coloured mutant obtained by Valadon & Heale, 1965. After about 6 weeks incubation on 2 % malt agar M5 develops the black melanoid pigment typical of the wild type (Heale & Isaac, 1964). To prevent the development of this pigment the stock cultures were subcultured once a month. M5·6 is one of these subcultures, it possesses the same carotenoids as the original M5, but in slightly different proportions.

The cultural conditions and the extraction and identification of the carotenoids were as described previously (Valadon & Heale, 1965). Authentic phytofluene was obtained from *Gerbera jamesonii* (Valadon, unpublished observation), neurosporene from *Neurospora crassa* (Haxo, 1949) and β-carotene and lycopene were provided by Dr O. Isler (Hoffmann-La Roche Ltd., Basle). β-zeacarotene and ζ-carotene were purified and identified according to the method of Simpson *et al.* (1963).

Some of the cultures were grown in the presence of β-ionone or methylheptenone vapours. This was achieved by dipping a cork into these liquids, and touching the cork surface lightly to the inner tip of the cotton wool plugs, which were then re-inserted into the mouths of the conical flasks (Simpson *et al.* 1964). DPA or 2-hydroxydiphenyl to be incorporated in the medium was dissolved in a few ml. methanol and this solution was then added to the culture medium.

Diphenylamine (DPA) has an absorption maximum in hexane at 282 mμ; Goodwin (1952) suggested that this interfered with the phytoene fraction in Phycomyces. Turian (1957) treated Neurospora cultures with 1/40,000 DPA + Fe^{2+} 0·2 mg./l. and found that there was no DPA left in the unsaponifiable fraction. Villoutreix (1960) however, found that in his DPA-treated culture of *Rhodotorula mucilaginosa*, DPA was possibly strongly fixed on to the organisms, and would therefore appear in the phytoene fraction. The phytoene fraction of our cultures of M5·6 when grown in presence of DPA showed a single maximum absorption peak at 282 mμ, corresponding to DPA. Phytoene was obtained pure by re-chromatography on MgO + celite by using purified hexane as developer, collecting 10 ml. portions of the eluate and reading these separately.

RESULTS

The pigments of *Verticillium albo-atrum* M5·6 are given in Table 1.

Use of inhibitors

β-ionone and methylheptenone vapours. Results obtained with these two compounds (Table 1) show much the same pattern, in that the phytoene content was increased considerably while γ-carotene, lycopene, and neurosporaxanthin were decreased; β-carotene was the only compound which behaved differently as it increased slightly with β-ionone and decreased with methylheptenone. The following carotenoids were identified for the first time in mutants of Verticillium: phytofluene, β-zeacarotene, ζ-carotene, neurosporene. The same amounts of phytofluene and ζ-carotene were obtained in both the treated cultures. Approximately twice as much β-zeacarotene and neurosporene were obtained in the methylheptenone cultures.

Table 1. *Effect of β-ionone and methylheptenone vapours on carotenoids of Verticillium albo-atrum* M 5·6 *(10-day cultures)*

	Treatment					
	Control		β-Ionone Carotenoids		Methylheptenone	
C₄₀ pigments	Amount (μg./g.)	%	Amount (μg./g.)	%	Amount (μg./g.)	%
Phytoene	32·5	16·1	130·5	64·0	90·0	45·6
Phytofluene	—	—	4·9	2·4	4·3	2·2
β-Carotene	17·7	8·6	21·2	10·4	13·6	6·9
β-Zeacarotene	—	—	7·2	3·5	14·1	7·1
ζ-Carotene	—	—	8·2	4·0	6·9	3·5
γ-Carotene	42·2	20·9	14·1	7·0	21·7	10·9
Neurosporene	—	—	4·6	2·3	9·3	4·7
Neo-lycopene A	6·6	3·3	—	—	—	—
Lycopene isomer?	—	—	5·6	2·7	2·4	1·2
Lycopene	23·2	11·5	1·5	0·7	2·2	1·2
Neurosporaxanthin	80·2	39·6	6·1	3·0	33·0	16·7
Total	202·4	100·0	203·9	100·0	197·5	100·0

Diphenylamine (DPA). Different concentrations of DPA were used; the results are recorded in Table 2. At a concentration of 1/200,000 DPA, phytoene was increased from 16 to 37 %; phytofluene, β-zeacarotene, ζ-carotene and neurosporene were identified. There was however, a decrease in β-carotene, γ-carotene, lycopene and neurosporaxanthin. At the higher concentration of 1/25,000 DPA, very much the same pattern was obtained: an inhibition of the production of the most unsaturated polyenes (β-, γ-carotene, lycopene, neurosporaxanthin) and a stimulation of the more saturated ones (phytoene, phytofluene, β-zeacarotene, ζ-carotene, neurosporene).

Table 2. *Effect of the concentrations of diphenylamine on carotenoids of Verticillium albo-atrum* M 5·6 *(10-day cultures)*

	Diphenylamine concentration			
	1/200,000		1/25,000	
	Carotenoids			
C₄₀ pigments	Amount (μg./g.)	%	Amount (μg./g.)	%
Phytoene	70·0	37·4	108·4	56·4
Phytofluene	2·8	1·5	3·3	1·7
β-Carotene	5·0	2·7	4·9	2·6
β-Zeacarotene	2·3	1·2	6·8	3·5
ζ-Carotene	2·1	1·2	2·5	1·3
γ-Carotene	32·4	17·3	18·4	9·7
Neurosporene	1·8	1·0	2·5	1·3
Lycopene isomer?	3·3	1·8	1·8	0·9
Lycopene	1·3	0·7	1·0	0·5
Neurosporaxanthin	65·9	35·2	42·5	22·1
Total carotenoids	186·9	100·0	192·1	100·0

Counteracting the effect of DPA

Slechta, Gabriel & Hoffmann–Ostenhof (1958) who worked with *Rhodotorula gracilis* found that inhibition by DPA was annulled when the organisms treated with DPA were transferred to a medium containing a nitrogen source. Goodwin *et al.* (1953) grew mycelium of *Phycomyces blakesleeanus* in DPA, washed it thoroughly, incubated it for 1 day in water, transferred to new media, and found that carotenoids appeared. With DPA treated *Rhodotorula mucilaginosa* cultures, carotenogenesis appeared only after the organisms had grown on a DPA-free medium and were transferred at least five times to new media. Villoutreix (1960) suggested that DPA is strongly fixed to the organisms and when DPA is diluted sufficiently to render it inactive, then carotenoids appear again.

In an attempt to annul the effect of DPA, in the hope of demonstrating the conversion of the more saturated polyenes into the most unsaturated ones, *Verticillium albo-atrum* M5·6 was grown in 40 p.p.m. DPA, and after 6 days the cultures were divided into three parts. The first part was analysed for its carotenoid contents (Table 3). The results as compared with strain M5·6 grown in the usual DPA-free medium (Table 3) showed the same pattern as before: an inhibition of the formation of the most unsaturated polyenes, a stimulation of the more saturated ones and the appearance of the four carotenes: phytofluene, β-zeacarotene, ζ-carotene, neurosporene.

The second portion of the culture was washed four times in $M/15$-KH_2PO_4, transferred to new DPA-free media containing peptone, and the carotenoids estimated after 10 days. The results (Table 3) show that the total amounts of carotenoids were much the same, with a high content of the C_{40} polyene phytoene (slightly higher than after 6 days) and a slight decrease in all the other carotenoids, thus suggesting that the inhibitory effect of DPA has hardly been affected.

Table 3. *Effect of diphenylamine (DPA) on Verticillium albo-atrum* M5·6

	Treatment of cultures									
	M5·6 (6-day cultures)		+1/25,000 DPA (6-day cultures)		After 6 days in ordinary medium, DPA added		After 6 days in DPA medium washed and placed in peptone medium		After 6 days in DPA medium washed and placed in 1 % glucose +M/15-KH₂PO₄	
	Carotenoids				Extraction after 10 days					
C_{40} pigments	Amount (µg./g.)	%	Amount (µg./g.)	%	Amount (µg./g.)	%	Amount (µg./g.)	%	Amount (µg./g.)	%
Phytoene	33·4	26·3	118·5	69·1	101·0	58·6	122·8	77·6	65·9	45·3
Phytofluene	—	—	3·0	1·7	trace	—	4·3	2·7	trace	—
β-Carotene	7·2	5·7	6·6	3·8	8·3	4·8	6·2	3·9	33·3	22·9
β-Zeacarotene	—	—	3·6	2·1	2·4	1·4	2·2	1·4	1·1	0·8
ζ-Carotene	—	—	2·8	1·6	2·2	1·3	1·7	1·1	0·4	0·3
γ-Carotene	8·2	6·5	6·6	3·8	13·1	7·6	4·8	3·0	17·9	12·3
Neurosporene	—	—	2·9	1·6	trace	—	0·7	0·4	0·4	0·3
Lycopene	5·5	4·3	5·2	3·0	3·3	1·9	3·4	2·1	3·6	2·5
Neurosporaxanthin	72·8	57·2	22·4	13·3	42·0	24·4	12·1	7·8	22·8	15·6
Total	127·1	100·0	171·6	100·0	172·3	100·0	158·2	100·0	145·4	100·0

The third portion of the culture was washed in M/15 phosphate buffer 4 times, incubated for one day in water and then transferred to a new medium of 1 % glucose in M/15-KH$_2$PO$_4$. These cultures were extracted after 10 days; the results (Table 3) show that phytoene and the other saturated polyenes were decreased while β-carotene, γ-carotene and neurosporaxanthin were increased.

Action of diphenylamine

Goodwin *et al.* (1953) found that adenylic acid and riboflavin annulled the inhibitory effect of DPA in *Phycomyces blakesleeanus* and suggested that DPA interfered with oxidative phosphorylation in that fungus. Turian (1957) suggested that this interference might only be a secondary effect since other organisms, *Mycobacterium phlei* (Goodwin, 1953) and *Rhodospirillum rubrum* (Goodwin & Osman, 1953) did not show this. With *Neurospora crassa*, Turian (1957) suggested that as DPA has strong anti-oxidation properties it inhibited the dehydrogenation of precursors of carotenoids. Rilling (1965) suggested that as DPA had the same molecular structure as that of the central part of phytofluene, it might act by binding with the enzyme (which would interact with the more saturated carotenoid to perform the dehydrogenation reaction) and in so doing inhibit the dehydrogenation of the carotenoid. The possibility exists then that if the more unsaturated pigments were already formed, these would not show a decrease.

The following experiment was therefore made.

Verticillium albo-atrum M5·6 was grown for 6 days in ordinary medium and then 1/25,000 DPA was added. The culture became paler in colour after 12 hr. After 4 more days, the carotenoids were extracted; the results given in Table 3. There was a slight increase in phytoene as compared to that in cultures grown in DPA for 10 days (Table 2), and only a trace of phytofluene and neurosporene. The β-carotene and lycopene contents were higher but the others were lower except ζ-carotene and neurosporaxanthin which were in more or less the same amounts. When, however, the carotenoid content of cultures grown in DPA-free culture for 6 days and then in DPA is compared with that of strain M5·6 after 6 days in ordinary DPA-free medium, one notices a high increase in phytoene, a slight increase in γ-carotene and a decrease in lycopene and neurosporaxanthin.

2-Hydroxydiphenyl. Villoutreix (1960) showed that 2-hydroxydiphenyl was an inhibitor of carotenogenesis in *Rhodotorula mucilaginosa*, that it did not interfere with growth and that its inhibition could be annulled fairly easily. Rilling (1965), however, did not find any effect of this compound on *Mycobacterium phlei*. Villoutreix's method was tried with *Verticillium albo-atrum* M5·6, in an effort to test whether 2-hydroxydiphenyl is inhibitory to Verticillium and if so, what pigments are formed once the inhibition is annulled. The cultures were grown in medium +10 p.p.m. 2-hydroxydiphenyl and after 7 days, one half was used for carotenoid determination (Table 4) while the other half was washed in cold water 4 times, suspended in M/15-KH$_2$PO$_4$ solution and incubated for a further 3 days, after which the carotenoids were extracted. The results (Table 4) showed that there was a decrease in phytoene, β-zeacarotene, ζ-carotene and neurosporene; β-carotene increased as did γ-carotene, lycopene and neurosporaxanthin after washing free from 2-hydroxydiphenyl, as compared to 7-day cultures containing 2-hydroxy-

diphenyl. 2-Hydroxydiphenyl at higher concentrations namely those used by Villoutreix (1960) of 4–5×10^{-5} inhibited strongly the growth of *V. albo-atrum* M5·6.

Table 4. *Effect of hydroxydiphenyl on carotenoids of*
Verticillium albo-atrum M5·6

	Treatment			
	10 mg./l. hydroxydiphenyl (7-day cultures)		After 7 days with hydroxydiphenyl washed and suspended in M/15-KH$_2$PO$_4$ for 3 days	
	Carotenoids			
Carotenoids	Amount (μg./g.)	%	Amount (μg./g.)	%
Phytoene	103·5	69·7	78·0	49·1
Phytofluene	—	—	—	—
β-Carotene	7·2	4·9	22·9	14·4
β-Zeacarotene	3·9	2·6	0·8	0·5
ζ-Carotene	1·7	1·1	0·6	0·4
γ-Carotene	5·5	3·7	12·8	8·1
Neurosporene	3·1	2·1	0·9	0·6
Lycopene	—	—	3·2	2·0
Neurosporaxanthin	23·5	15·9	39·5	24·9
Total	148·4	100	158·7	100

DISCUSSION

β-Ionone, a compound which is theoretically capable of providing terminal groups to specific carotenoid molecules, was found to markedly increase β-carotene production in Phycomyces by Mackinney *et al.* (1952). Reyes (1963; quoted by Simpson *et al.* 1964) showed that β-ionone stimulated carotenoid and sterol formation and suggested that the effect was one of an inhibition of a negative feedback mechanism acting at the pathway level of the phosphorylated derivations of mevalonic acid. An increase in β-carotene was not obtained by Nakayama *et al.* (1957) with *Syncephalastrum racemosum* and Simpson *et al.* (1964) with *Rhodotorula glutinis*. The varied effects shown by β-ionone may be explained on the grounds that it is difficult to control the amount of β-ionone effectively present in the cultures. At low concentrations β-ionone appears to stimulate β-carotene synthesis and at higher concentrations it is inhibitory. In *Verticillium albo-atrum* M5·6, we found a slight increase in β-carotene and at the same time a substantial increase in the more saturated compounds and a decrease in the most unsaturated compounds except β-carotene. Similar results were obtained with methylheptenone. Nakayama *et al.* (1957) showed in Phycomyces that methylheptenone increased phytoene, phytofluene and ζ-carotene significantly while β-carotene was only slightly increased. In *Syncephalastrum racemosum*, however, while the phytoene was increased significantly the β-carotene decreased appreciably. Simpson *et al.* (1964) obtained very much the same results as with β-ionone: stimulation in the formation of phytoene, phytofluene, ζ-carotene, neurosporene and β-zeacarotene and considerable

diminution of β-carotene, torulin and torularhodin. In fact, Simpson *et al.* seem to be the only workers to have obtained quite such similar results with both compounds. Our results too, with the two compounds, resembled theirs except that there was stimulation of β-carotene with β-ionone, and inhibition of it with methylheptenone; associated with this difference in β-carotene formation, there was much less γ-carotene (14·1 μg./g.) with β-ionone than with methylheptenone (21·7 μg./g.). Our results would fit in with the hypothesis that the inhibiting compounds block dehydrogenation steps in the Porter–Lincoln pathway, causing the accumulation of the more saturated intermediates. The different amounts of β-carotene may be due to an additional block between the step γ- $\rightarrow$ β-carotene.

With methylheptenone, β-ionone and with DPA all the intermediates of the Porter & Anderson (1962) hypothesis (Fig. 1) from phytoene to β-carotene, including lycopene and β-zeacarotene have been isolated with M5·6 cultures. The presence of these C_{40} polyenes does not however prove sequential pathways from phytoene to β-carotene. With DPA (40 mg./l.) the cultures were still slightly coloured and when washed, incubated in water for one day and transferred to a medium containing 1 % glucose in M/15-KH_2PO_4, the more unsaturated polyenes namely β-, γ-carotene and neurosporaxanthin seem to be formed at the expense of the more saturated ones, phytoene, phytofluene, ζ-carotene, neurosporene and β-zeacarotene. Lycopene, however, is increased from 0·5 to 2·5 %, and this might seem to suggest that γ-carotene is formed via β-zeacarotene rather than through lycopene.

With hydroxydiphenyl on *Rhodotorula mucilaginosa*, Villoutreix (1960) showed that most of the more saturated polyenes except phytofluene were identified. When grown on 50 μg hydroxydiphenyl/ml., colourless cultures were obtained which still contained phytoene, ζ-carotene and neurosporene. 24 hr after removal of the inhibitor, β- and γ-carotene appeared as well as torulene and torularhodin, but there were still no signs of any phytofluene. The absence of phytofluene made him suggest that the step phytoene $\rightarrow$ phytofluene was doubtful and similarly the step phytofluene $\rightarrow$ ζ-carotene. Our results with the same inhibitor agree very closely with his in that no phytofluene was obtained in cultures treated with hydroxydiphenyl. However, when the effect of the inhibitor was removed there was an increase in β-, γ-carotene, lycopene and neurosporaxanthin presumably at the expense of phytoene, β-zeacarotene, ζ-carotene and neurosporene. With the other inhibitor DPA, we have obtained phytofluene and this tends to suggest that phytofluene may take part in the steps phytoene $\rightarrow$ phytofluene $\rightarrow$ ζ-carotene, though not identified in hydroxydiphenyl inhibited cultures. It is possible that phytofluene was formed and used up rapidly and therefore would not have appeared in the cultures. Our results are, then, consistent with the proposed sequential pathway as suggested by Porter & Anderson (1962), and also that γ- and therefore β-carotene are synthesized through β-zeacarotene as suggested by Simpson *et al.* (1964), Davies *et al.* (1963) rather than through lycopene.

Our cultures after carotenoid synthesis had started became visibly paler after DPA was added. However, this seems to be due only to loss in amount of neurosporaxanthin by about a half. The more unsaturated pigments that had already been formed do not show a decrease, in fact, a slight increase, and this supports the suggestion made by Turian (1957) and Rilling (1965) that DPA may act in inhibiting the dehydrogenation of precursors of carotenoids. However, it is also well known

that growth of fungi is affected by DPA and it is very probable that metabolism of carotenoids may not be the only system affected (Villoutreix, 1960), even though Olson & Knizley (1962) showed that the effect of DPA is apparently restricted to carotenoid biosynthesis, as neither sterols nor fatty acids seem to be affected.

2-hydroxydiphenyl too seems to show different effects on different microorganisms. The growth of *Rhodotorula mucilaginosa* is not affected at concentrations of 50 μg./ml. (Villoutreix, 1960) while it acts as a good inhibitor of carotenoid synthesis in this organism. Rilling (1965) finds it without any effect on the carotenoids of *Mycobacterium phlei* and in *Verticillium albo-atrum*, no growth is observed at 50 mg./l. while at 10 mg./l. it affects carotenoid synthesis.

These examples demonstrating the different effects of the same carotenoid inhibiting compounds on different organisms emphasize the impossibility at least at the moment of obtaining any complete and/or unified picture of carotenoid synthesis in micro-organisms.

REFERENCES

CHICHESTER, C. O., WONG, P. S. & MACKINNEY, G. (1954). On the biosynthesis of Carotenoids. *Plant Physiol.* **29**, 238.

DAVIES, B. H. (1961). The biosynthesis of carotenoids of *Rhizophlyctis rosea. Biochem. J.* **80**, 48 P.

DAVIES, B. H., VILLOUTREIX, J., WILLIAMS, R. J. H. & GOODWIN, T. W. (1963). The possible role of β-zeacarotene in carotenoid synthesis. *Biochem. J.* **89**, 96 P.

GOODWIN, T. W. (1952). Identification of the minor polyene components of the fungus *Phycomyces blakesleeanus* and a study of their synthesis under various cultural conditions. *Biochem. J.* **50**, 550.

GOODWIN, T. W. (1953). The biogenesis of carotenoids. *J. Sci. Fd Agric.* **5**, 209.

GOODWIN, T. W. & OSMAN, H. G. (1953). General cultural conditions controlling carotenoid (spirilloxanthin) synthesis in the photosynthetic bacterium *Rhodospirillum rubrum. Biochem. J.* **53**, 541.

GOODWIN, T. W., JAMIKORN, M. & WILLMER, J. S. (1953). Further observations concerning the action of diphenylamine in inhibiting the synthesis of β-carotene in *Phycomyces blakesleeanus. Biochem. J.* **53**, 531.

HAXO, F. (1949). Studies on the carotenoid pigments of Neurospora. *Arch. Biochem.* **20**, 400.

HEALE, J. B. & ISAAC, I. (1964). Dark pigment formation in *Verticillium albo-atrum. Nature, Lond.* **202**, 412.

KRZEMINSKI, L. F. & QUACKENBUSH, F. W. (1960). Incorporation of C^{14}-labelled compounds into carotenes by *Neurospora crassa. Arch. Biochem. Biophys.* **88**, 287.

MACKINNEY, G., NAKAYAMA, T., BUSS, C. D. & CHICHESTER, C. O. (1952). Carotenoid production in Phycomyces. *J. Am. chem. Soc.* **74**, 3456.

NAKAYAMA, T., CHICHESTER, C. O., LUKTON, A. & MACKINNEY, G. (1957). Phytoene production in Phycomyces. *Arch. Biochem. Biophys.* **66**, 310.

OLSON, J. A. & KNIZLEY, J. H. (1962). The effect of diphenylamine on carotenoid, sterol and fatty acid synthesis in *Phycomyces blakesleeanus. Arch. Biochem. Biophys.* **97**, 138.

PORTER, J. W. & ANDERSON, D. G. (1962). The biosynthesis of carotenes. *Arch. Biochem. Biophys.* **97**, 520.

PORTER, J. W. & LINCOLN, R. E. (1950). The mechanism of carotene biosynthesis. *Arch. Biochem.* **27**, 390.

RILLING, H. C. (1965). A study of inhibition of carotenoid synthesis. *Arch. Biochem. Biophys.* **110**, 39.

RÜEGG, R., SCHWIETER, U., RYSER, G., SCHUDEL, P. & ISLER, O. (1961). Synthesen in der Carotenoid-Reihe. *Helv. chim. Acta* **44**, 994.

SIMPSON, K. L., NAKAYAMA, T. O. M. & CHICHESTER, C. O. (1964). Biosynthesis of yeast carotenoids. *J. Bact.* **88**, 1688.

SLECHTA, L., GABRIEL, O. & HOFFMAN-OSTENHOF, O. (1958). Separation of carotenoid synthesis from fat synthesis in *Rhodotorula gracilis* by diphenylamine. *Nature, Lond.* **181**, 268.

TURIAN, G. (1957). Recherches sur l'action anticaroténogène de la diphénylamine et ses conséquences sur la morphogenèse reproductive chez Allomyces et Neurospora. *Physiol. Plant.* **10**, 667.

TURIAN, G. & HAXO, F. (1954). Minor polyene components in the sexual phase of *Allomyces javanicus*. *Bot. Gaz.* **115**, 254.

VALADON, L. R. G. & HEALE, J. B. (1965). Carotenoids in mutants of *Verticillium albo-atrum. J. gen. microbiol.* **41**, 341.

VILLOUTREIX, J. (1960). *Sur les Caroténoïdes d'une Levure Rouge: Rhodotorula mucilaginosa (Jorg) Harrison.* Nancy: Imprimerie Grandville.

ZALOKAR, M. (1954). Studies on biosynthesis of carotenoids in *Neurospora crassa. Arch. Biochem. Biophys.* **50**, 71.

The Morphogenetic Effect of Sulphur-Containing Amino Acids, Glutathione and Iodoacetic Acid on *Sclerotium rolfsii* Sacc.

By I. CHET, Y. HENIS

Faculty of Agriculture, The Hebrew University

AND R. MITCHELL

Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel

SUMMARY

The sulphur-containing amino acids L-cysteine, L-cystine, L-methionine, L-homocysteine thiolactone, L-homocystine and glutathione at 10^{-4} to 10^{-5}M inhibited sclerotial formation without inhibition of mycelial growth and induced basidia formation in the fungus *Sclerotium rolfsii* Sacc. Higher concentrations inhibited linear growth and production of sclerotia. The effect of L-cysteine was competitively antagonized by iodoacetic acid at a molar ratio of 30:1. Iodoacetic acid induced sclerotial formation at 3×10^{-5} to 10^{-4}M, sclerotia being produced in circles around inoculation point. The possible role of –SH groups in the morphogenesis of *S. rolfsii* is discussed.

INTRODUCTION

Sclerotia are bodies produced by some septate fungi, they consist of an outer layer of thick-walled usually darkly pigmented cells which surround a mass of thinner-walled closely interwoven hyphae (Townsend & Willets, 1954). Because of their high resistance to chemical and biological degradation, many pathogenic fungi survive in soil as sclerotia. In spite of their biological and ecological significance, little is known about the factors which affect the production of sclerotia. The formation of microsclerotia by *Verticillium albo-atrum* is affected by light and an unidentified diffusible factor (Brandt, 1964). The production of sclerotia by *Sclerotium rolfsii* has been correlated with genetic processes (Higgins, 1927), growth under suboptimal conditions (Boyle, 1961) or inhibition of the growth of mycelial tips (Henis, Chet & Avizohar-Hershenzon, 1965; Wheeler & Waller, 1965). Attempts to control the production of sclerotia by *S. rolfsii* by sulphur-containing amino acids and related compounds led to the finding that these compounds not only affect sclerotidal production, but may induce the appearance of the sexual stage of this fungus, which is rather rare (West, 1947).

METHODS

The strain of *Sclerotium rolfsii* Sacc. used throughout these experiments was isolated from sugar beet. It was grown on a chemically defined medium solidified with agar prepared according to Joham (1943) and was composed of (g./l.): glucose,

40; K_2HPO_4, 0·7; NH_4NO_3, 1; KCl, 0·15; $MgSO_4.7H_2O$, 0·2; $FeSO_4$, 0·002; $MnCl_2$, 0·002; $ZnSO_4$, 0·002; thiamine chloride, 0·0001; Bacto agar (Difco), 20; in distilled water 1 l.; adjusted to pH 7·0. The medium autoclaved for 15 min. at 121°.

The compounds to be tested were prepared at tenfold concentrations, sterilized by filtration through a Seitz filter and 1 ml. samples mixed with 9 ml. melted agar medium which was kept at 45°. Inocula were prepared from 3-day cultures, grown on defined medium agar at 30°. Agar discs 0·5 cm. in diameter, covered with fungal mycelium, were cut by a sterile cork borer and placed at the centre of the defined medium agar plates containing the tested compounds. The incubation temperature was 30°. After incubation, sclerotia were collected and counted. Hyphal weight was determined by melting the agar, washing the remaining mycelium with hot water, drying it in the oven to constant weight in aluminium planchets at 60°, and weighing. Linear growth was determined by measuring the diameter of the growing colonies.

RESULTS

Effect of sulphur-containing amino acids and related compounds on the formation of sclerotia and mycelial growth of Sclerotium rolfsii

Plates containing defined medium agar with the tested compounds were inoculated with mycelial discs and incubated for 2 weeks at 30°. The sclerotia were collected and counted, hyphal weight and linear growth determined as described. The results are summarized in Fig. 1 and Table 1. L-Cysteine, L-cystine, L-methionine, L-homocysteine thiolactone, L-homocystine and glutathione at 10^{-4} to 10^{-5} M inhibited sclerotia formation without affecting mycelial weight and its linear growth. Although mycelial weight was not affected significantly at concentrations greater than 10^{-4} M, both sclerotial formation and linear growth of mycelium were inhibited, the mycelium being denser and thicker than that of the control (Pl. 1, figs. 1, 2). Little inhibition of sclerotial formation and no effect on mycelial weight and linear growth were observed with β-mercaptoethanol, 2,3-dimercaptopropanol and thioglycollic (mercapto-acetic) acid, at 10^{-4} to 10^{-5} M. Higher concentrations (up

Table 1. *The effect of some sulphur-containing compounds on linear growth and mycelial weight of Sclerotium rolfsii*

Incubation time: 7 days. Each value represents an average of 10 replicates.

| | Concentration of sulphur compound (M) | | | | | |
| | 10^{-4} | | 10^{-3} | | 10^{-2} | |
Compound	Colony diameter (mm.)	Dry weight (mg./plate)	Colony diameter (mm.)	Dry weight (mg./plate)	Colony diameter (mm.)	Dry weight (mg./plate)
L-Methionine	90*	85	85	27	65	25
L-Cysteine	90	130	71	245	42	117
L-Cystine	90	25	74	69	46	60
Glutathione	90	155	90	126	57	60
L-Homocystine	90	93	90	55	—	—
L-Homocysteine thiolactone	90	154	90	130	71	137
Control	90	145				

* Colony diameter equal to that of the Petri dish at time of estimation.

to 10^{-2}M) inhibited both linear growth and sclerotial formation. No inhibition was observed at these concentrations with L-histidine, L-tyrosine, glycine, L-threonine, L-alanine, L-phenylalanine, L-asparagine, L-lysine or L-serine. In effective treatments at concentrations of 10^{-5} to 10^{-3}M formation of sclerotia was only temporarily arrested, lasting for 2 or 3 weeks; at 10^{-2}M both restriction of mycelial linear growth and permanent inhibition of sclerotial formation occurred.
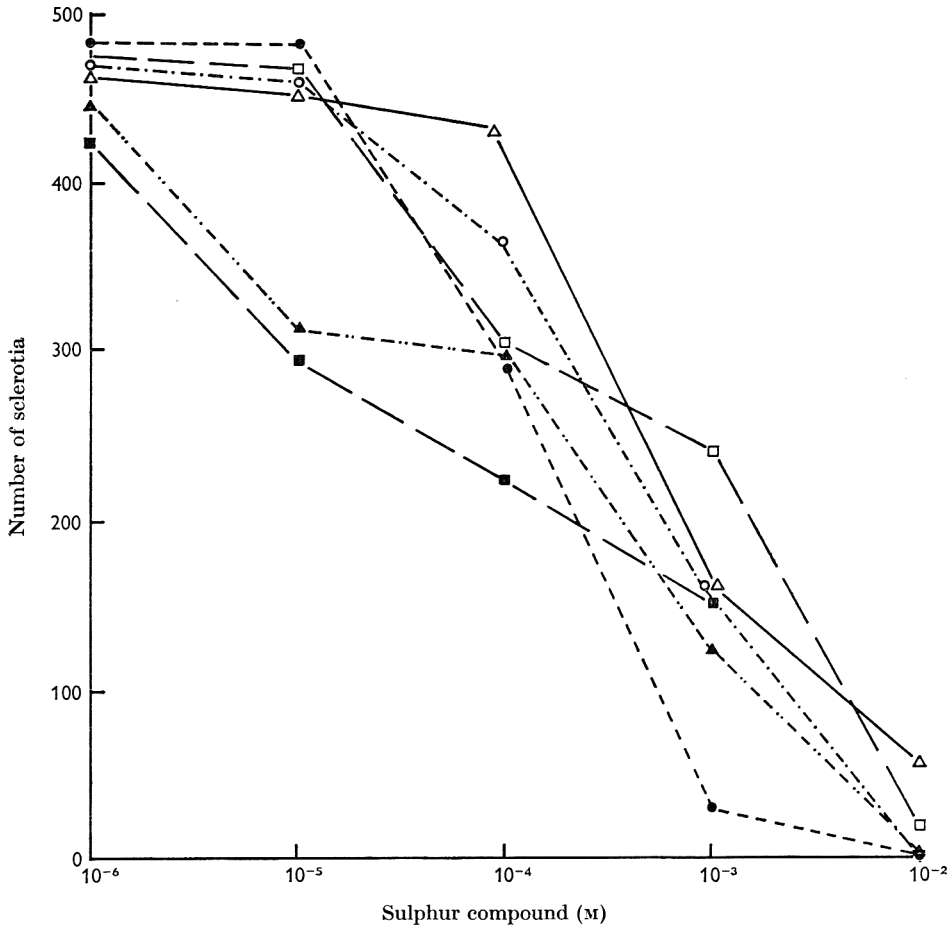
Fig. 1. Effect of some sulphur-containing compounds on production of sclerotia by *Sclerotium rolfsii*. $---●---$, L-Cysteine; $-·-·-○-·-·-$, L-Cystine; $—■—$, L-homocystine; $—□—$, L-methionine; $—△—$, L-homocysteine thiolactone; $···—▲—···$, glutathione. Control: 490 sclerotia. Incubation: 7 days at 30°. Each value represents an average of 10 replicates.

Effect of sulphur-containing amino acids and related compounds
on the formation of the sexual stage of Sclerotium rolfsii

In general, compounds which inhibited sclerotial formation without inhibiting linear mycelial growth stimulated the appearance of the sexual stage of *Sclerotium rolfsii* (Fig. 2). Basidia were formed mainly on side walls of the Petri dishes, the mycelium in these areas becoming very dense, yellow-cream in colour and typically

odorous. The cream-coloured dense mycelium was sectioned by means of an ice microtome (Reichert Optical Works, Austria) to a width of 10 μ and examined by a phase-contrast microscope. Basidia, sterigmata and basidiospores typical of the perfect stage of *S. rolfsii* (West, 1947) were observed.
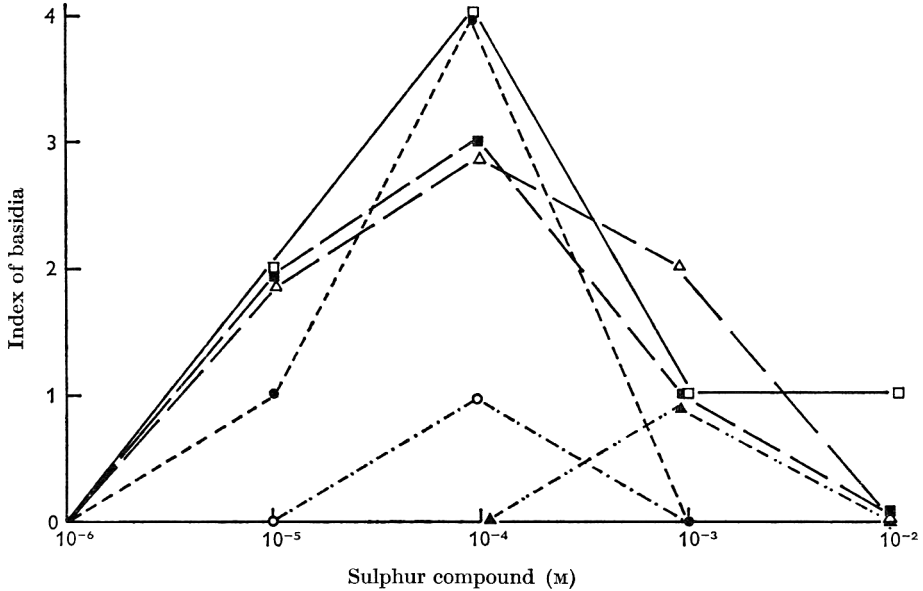
Fig. 2. Effect of some sulphur-containing compounds on production of basidia by *Sclerotium rolfsii*. Index of basidia is based on an arbitrary scale: from zero, no basidia, to 4, maximal amount of basidia. Legend, conditions and replicates, as for Fig. 1. Control: no basidia.

Effect of iodoacetic acid on the production of sclerotia by Sclerotium rolfsii

Because of the inhibitory effect of sulphydryl-containing amino acids on the production of sclerotia by *Sclerotium rolfsii*, it seemed of interest to examine the possible stimulatory effect of –SH antagonists on production of sclerotia. The effect of iodoacetic acid, L-cysteine and of mixtures of these compounds on sclerotial production by *S. rolfsii* is shown in Pl. 1. The optimal concentration of iodoacetic acid for sclerotial formation was 10^{-4}M. Higher concentrations (5×10^{-3}M or more) inhibited mycelial growth. Whereas the total numbers of sclerotia produced on plates containing iodoacetic acid were not significantly higher than that of the controls, the sclerotia were produced earlier (30 hr as compared with 4–5 days), and at the mycelial edges only, in concentric circles around the initial inoculum (Pl. 1, fig. 3), several circles of sclerotia, produced at intervals, being formed around the inoculum.

Both inhibition of mycelial growth and concentric production of sclerotia by iodoacetic acid was prevented by the addition of L-cysteine to the medium. At effective concentrations, a molar ratio of 30:1 of L-cysteine:iodoacetic acid was required for the complete mutual elimination of their effects.

DISCUSSION

Environmental factors which have been reported to affect sporulation, spore germination and sclerotial formation in fungi, include CO_2, N_2, vitamins, pH value, light, temperature, humidity, and 'atmospheric conditions' (Baldwin & Rusch, 1965). The effect of sulphur-containing amino acids on vegetative form changes in fungi has been shown by Nickerson & Falcone (1956), Nickerson (1963) and Nickerson & Bartnicki-Garcia (1964). A stimulatory effect of sulphur-containing amino compounds on the formation of the perfect stage of Aphanomyces root rot has been also shown (Papavizas & Davey, 1963). However, no information is available about the effect of sulphur compounds on the formation of sclerotia and basidia in fungi.

In testing the effect of amino acids on the formation of sclerotia and basidia in *Sclerotium rolfsii* only the sulphur-containing amino acids, especially L-cysteine, were active. Their addition to the medium at 10^{-4} to 10^{-5}M resulted in decreased formation of sclerotia by *S. rolfsii*, without affecting mycelial weight and its linear growth, and in the induction of the production of basidia. However, other –SH-containing compounds such as β-mercaptoethanol, 2,3-dimercaptopropanol and thioglycollic acid were much less active. The morphogenetic effect of sulphur-containing amino acids and glutathione on the formation of sclerotia and basidia, does not necessarily mean that the same mechanism is involved.

If the intracellular concentration of –SH groups is a critical factor in the morphogenesis of *Sclerotium rolfsii* then it might be possible to induce sclerotial formation by a –SH antagonist such as iodoacetic acid. Indeed, the addition of 10^{-4}M iodoacetic acid resulted in stimulation of sclerotial formation within 30 hr from inoculation time. However, induction was limited to hyphal tips in the periphery of the fungal colony, the sclerotia forming a circle around the inoculation point. Moreover, the linear growth of the vegetative mycelium continued and new circles of sclerotia were formed around the first one (Pl. 1, fig. 3). Thus, iodoacetic acid enhanced sclerotial formation and changed the distribution of sclerotia on the agar plate. These phenomena may be connected with specific concentrations of iodoacetic acid at the mycelial tips, which result in a lower intracellular concentration of –SH groups. It seems now that inhibition of linear growth *per se* does not induce sclerotial formation, since both L-cysteine and iodoacetic acid inhibited linear mycelial growth at 10^{-2}M and 5×10^{-3}M, respectively; but whereas L-cysteine inhibited sclerotial formation as well, iodoacetic acid induced it.

Iodoacetic acid and L-cysteine competitively antagonized each other at a ratio of 1:30. This high ratio may be due to differences in permeability or in reaction rate inside the hyphal cells. The rate of reaction between these compounds at the pH value of the growth medium (7·0) is relatively slow (Cecil & McPhee, 1959).

The perfect stage of *Sclerotium rolfsii* Sacc. (*Pellicularia rolfsii*) is rather rare (West, 1947) and no information is available about the factors involved in its formation. We believe that this is the first instance of a stimulation of the appearance of the perfect basidial stage of this fungus on an artificial growth medium.

REFERENCES

Baldwin, H. H. & Rusch, H. P. (1965). The chemistry of differentiation in lower organisms. *A. Rev. Biochem.* **34**, 565.

Boyle, L. W. (1961). The ecology of *Sclerotium rolfsii* with emphasis on the role of saprophytic media. *Phytopathology* **51**, 117.

Brandt, W. H. (1964). Morphogenesis in Verticillium; a self-induced, non-hereditary variation in colour form. *Am. J. Bot.* **51**, 8.

Cecil, R. & McPhee, J. R. (1959). The sulfur chemistry of proteins. *Protein Chem.* **14**, 255.

Henis, Y., Chet, I. & Avizohar-Hershenzon, Z. (1965). Nutritional and chemical factors involved in mycelial growth and production of sclerotia by *Sclerotium rolfsii* in artificial medium and amended soil. *Phytopathology* **55**, 87.

Higgins, B. B. (1927). Physiology and Parasitism of *Sclerotium rolfsii* Sacc. *Phytopathology* **17**, 417.

Joham, H. E. (1943). *A nutritional study of the fungus Sclerotium rolfsii.* M.Sc. thesis. A and M College of Texas.

Nickerson, W. J. (1963). Symposium on biochemical bases of morphogenesis in fungi. IV. Molecular bases of form in yeasts. *Bact. Rev.* **27**, 305.

Nickerson, W. J. & Bartnicki-Garcia, S. (1964). Biochemical aspects of morphogenesis in algae and fungi. *A. Rev. Plant Physiol.* **15**, 327.

Nickerson, W. J. & Falcone, G. (1956). Identification of protein disulfide reductase as a cellular division enzyme in yeasts. *Science* **124**, 722.

Papavizas, G. C. & Davey, C. B. (1963). Effect of sulfur containing amino compounds and related substances on *Aphanomyces* root rot of peas. *Phytopathology* **53**, 109.

Townsend, B. B. & Willetts, H. J. (1954). The development of sclerotia of certain fungi. *Trans. Br. mycol. Soc.* **37**, 213.

West, E. (1947). *Sclerotium rolfsii* Sacc. and its perfect stage on climbing fig. *Phytopathology* **37**, 67.

Wheeler, B. E. J. & Waller, J. M. (1965). The production of sclerotia by *Sclerotium rolfsii*. II. The relationship between mycelial growth and initiation of sclerotia. *Trans. Br. mycol. Soc.* **48**, 303.

EXPLANATION OF PLATE

Plate 1. Effect of L-cysteine and iodoacetic acid on *Sclerotium rolfsii*.

Fig. 1. Normal growth of *S. rolfsii* on defined medium agar after incubation for 10 days at 30°. Brown sclerotia are especially abundant at the edges of the Petri dish. × 0·66.

Fig. 2. Growth of *S. rolfsii* in the presence of 10^{-2}M-L-cysteine. Mycelium is more dense than that seen in fig. 1. Only two sclerotia are seen; linear growth is significantly restricted. × 0·66.

Fig. 3. Effect of 10^{-4}M-iodoacetic acid on growth pattern of *S. rolfsii*. Mycelium not affected, but sclerotia are formed in circles around the inoculum and are bigger than those seen in fig. 1. × 0·66.

Fig. 4. Mutual inhibition of the effects of L-cysteine (10^{-4}M) and iodoacetic acid (3×10^{-5}M). A normal growth of *S. rolfsii* is seen. × 0·66.
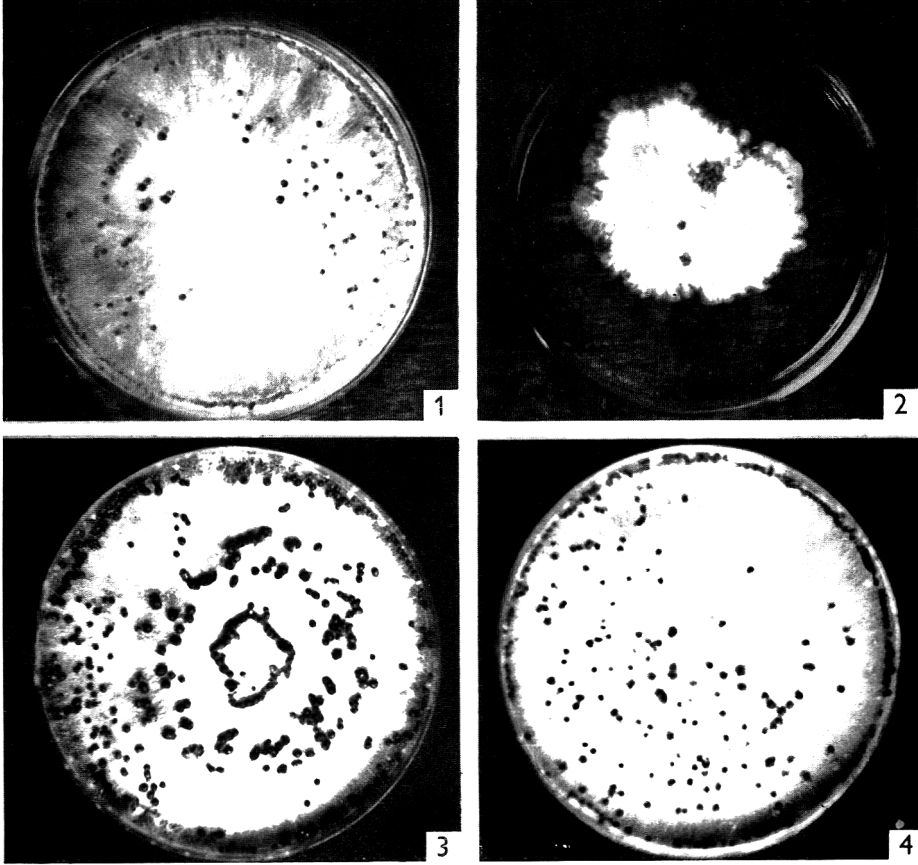
The Influence of Temperature and Growth Rate on the Quantitative Relationship Between Potassium, Magnesium, Phosphorus and Ribonucleic Acid of *Aerobacter aerogenes* Growing in a Chemostat

By J. W. DICKS and D. W. TEMPEST

Microbiological Research Establishment, Porton Down, Wiltshire

SUMMARY

The variations in potassium, magnesium and phosphorus contents of *Aerobacter aerogenes* grown in a chemostat, were measured as functions of the RNA content of the organisms. The RNA content was varied by altering temperature at a fixed dilution rate under potassium-limiting conditions, and by varying the growth rate at a fixed temperature under conditions of both magnesium- and phosphate-limitation. Changes in RNA were accompanied by corresponding changes in these other cellular components such that a molar stoichiometry close to 1:4:5:8 for magnesium, potassium, RNA (nucleotide) and phosphorus, respectively, was maintained. The only significant deviation from this ratio was observed in phosphate-limited organisms at low growth rates; these organisms possessed considerable amounts of carbohydrate. It is suggested that potassium, magnesium and phosphate are implicated in polysaccharide synthesis, thus changing the quantitative relationship between these three components and RNA previously observed. The results support the suggestion that most of the potassium in growing *A. aerogenes* is required to maintain the functional state of ribosomal particles.

INTRODUCTION

A wide range of growing cells require potassium and accumulate it to high intracellular concentrations (Steinbach, 1962; Lester, 1958; Rouf, 1964). The importance of potassium in certain enzyme-catalysed reactions (Dixon & Webb, 1964), particularly those which involve phosphate transfer (Lardy, 1951) may explain its frequently reported participation in carbohydrate metabolism (Pulver & Verzar, 1940; Roberts, Roberts & Cowie, 1949; Eddy & Hinshelwood, 1951; Duguid & Wilkinson, 1954) and respiration (Krebs, Whittam & Hems, 1957; Perry & Evans, 1961; Blond & Whittam, 1965). It seemed justifiable, however, to inquire whether these catalytic roles demand the presence of potassium in growing cells in concentrations which make it the major metal nutrient. We suggested an alternative explanation (Dicks & Tempest, 1966; Tempest, Dicks & Hunter, 1966), derived from an investigation of the growth of *Aerobacter aerogenes* in a chemostat under conditions of potassium-limitation. A growth rate-independent molar stoichiometry between the magnesium, potassium, RNA (nucleotide) and phosphorus contents of the organism of approximately 1:4:5:8 was interpreted in terms of potassium participation in the maintenance of a functional configuration of ribosomal structures. At present,

this hypothesis is based on evidence derived from the investigation of a single species of bacterium grown under one type of growth limitation. If the suggestion is tenable, then it might be anticipated that the described stoichiometry exists in many growing cells, regardless of the growth conditions. The aim of the work described in the present paper was to extend our observations on the same organism growing at different temperatures and under different growth limitations.

METHODS

Organism. Aerobacter aerogenes, NCTC 418, maintained by monthly subculture on tryptic meat digest agar slopes, was used throughout.

Growth conditions. Cultures were grown in 0·5 l. chemostats of the type described by Herbert, Phipps & Tempest (1965), in which the pH value (6·5 ± 0·1) and temperature (35°, except when stated otherwise) were each controlled automatically. Foaming of the cultures was diminished by the regular addition of 0·1–0·2 ml. Polyglycol P–2000 antifoam (Dow Chemical Co., Midland, Michigan, U.S.A.).

The K^+-limited culture medium was identical to that previously described by Tempest et al. (1966). The Mg^{2+}-limited culture medium contained: $(NH_4)H_2PO_4$, $4·5 \times 10^{-2}$ M; $(NH_4)_2SO_4$, $2·5 \times 10^{-2}$M; Na_2HPO_4, $5·0 \times 10^{-3}$M; K_2SO_4, $1·0 \times 10^{-3}$M or $2·0 \times 10^{-3}$M; citric acid, $1·0 \times 10^{-3}$M: $MgCl_2$, $2·5 \times 10^{-4}$M; $CaCl_2$ and $FeCl_3$, each $1·0 \times 10^{-4}$M; $MnCl_2$, $2·5 \times 10^{-5}$M; $CuCl_2$ and Na_2MoO_4, each $5·0 \times 10^{-6}$M; glycerol, 30 mg./ml. The PO_4^{3-}-limited culture medium contained $(NH_4)_2SO_4$, $5·0 \times 10^{-2}$M; Na_2HPO_4, $2·0 \times 10^{-3}$M; K_2SO_4, $1·5 \times 10^{-3}$M or $2·5 \times 10^{-3}$M; citric acid, $1·0 \times 10^{-3}$M; $MgCl_2$, $1·25 \times 10^{-3}$M; the Ca^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+} and molybdate concentrations, and the glycerol content, were identical to those described for the Mg^{2+}-limited medium.

All media were prepared in 20 l. volumes by using distilled water which had passed through a mixed-bed ion-exchange resin. The pH values of Mg^{2+}- and K^+-limited media were within the range pH 5·20–5·30, and of PO_4^{3-}-limited media within the range pH 3·20–3·30 after autoclaving (121°, 30 min.). Adjustment to pH 6·5 was attained in the culture vessel by the operation of the automatic pH control unit.

Samples, not exceeding 25 ml. (5 % of the culture volume), were taken directly from the culture vessel for analysis.

Analytical procedures. Bacterial concentration (equiv. mg. dry weight organisms/ml. culture), total cellular RNA and carbohydrate were estimated according to methods previously described by Tempest, Hunter & Sykes (1965). Total phosphorus was determined by the method of King (1951) after the bacteria has been digested with 60 % (w/v) perchloric acid. Intracellular magnesium was determined by a modification (Tempest & Strange, 1966) of the Titan Yellow method of Gardner (1946).

Identification of glycogen. The methods of Strange, Dark & Ness (1961) were employed for the extraction and identification of glycogen from PO_4^{3-}-limited organisms grown at a dilution rate of 0·09 hr⁻¹. Briefly, the procedure involved water-extraction of disrupted organisms, treatment of the extract with cold trichloracetic acid (TCA) to a final concentration of 2·5 % (w/v) to remove protein and nucleic acids, and then precipitation of glycogen from the neutralized dialysed solution with ethanol. After washing in ethanol and drying, the isolated material

gave a red colour with iodine and readily dissolved in water to an opalescent solution. It contained 66 % carbohydrate which, after acid hydrolysis and chromatographic analysis (using the spray reagents of Trevelyan, Proctor & Harrison (1950), and Partridge (1949)) was shown to consist entirely of glucose.

Potassium determinations were performed according to the method described by Tempest *et al.* (1966), in which the bacterial potassium content is derived by subtracting the residual potassium in the culture supernatant from the total potassium in the culture, after estimation by flame photometry. This method is facilitated by the virtually complete uptake of potassium under K^+-limiting conditions. However, when another component of the medium limits growth, potassium must be supplied in excess of requirement. Since the accuracy of this particular determination is dependent upon subtracting a small value from a relatively large one, the potassium content of Mg^{2+}- and PO_4^{3-}-limited culture media has been maintained at a value which ensures its presence in excess at all rates of growth without significantly decreasing the sensitivity of the analytical procedure.

Viability measurements were made by the slide culture technique of Postgate, Crumpton & Hunter (1961). Under all conditions of growth reported in this paper, the degree of viability exceeded 90 %.

<div align="center">RESULTS</div>

<div align="center">*Effect of temperature on the steady state composition of*
K^+-limited organisms growing at a fixed dilution rate</div>

If the growth rate-dependent variations in magnesium, potassium and phosphorus contents of *Aerobacter aerogenes* are connected primarily with the changes in RNA content of the organisms (Dicks & Tempest, 1966; Tempest *et al.* 1966), then the changes in RNA content which accompany different incubation temperatures at a fixed growth rate (Tempest & Hunter, 1965) should also be accompanied by changes in the potassium, magnesium and phosphorus contents of the organisms.

The influence of changes in the incubation temperature on K^+-limited cultures of *Aerobacter aerogenes*, growing at a dilution rate of 0·2 hr^{-1}, is shown in Table 1. Decrease in temperature from 35° to 25° caused a 36 % increase in bacterial RNA content; increases in bacterial potassium, phosphorus and magnesium were 33 %,

<div align="center">Table 1. *Analysis of K^+-limited Aerobacter aerogenes* NCTC *418 grown*
at different temperatures</div>

Duplicate analyses were done on samples collected on different days during 'steady state' growth at a dilution rate of 0·20 ± 0·01 hr^{-1}.

Temp. (°)	Yield (g. dry bacteria/g. K^+)	K^+	Mg^{2+} (g./100 g. dry bacteria)	P	RNA
35	97·1	1·03	0·157	1·68	11·6
35	93·5	1·07	0·160	1·84	12·6
30	84·8	1·18	0·156	1·90	13·1
30	82·7	1·21	0·173	1·95	13·5
25	70·5	1·42	0·187	2·33	16·6
25	72·5	1·38	0·189	2·28	16·3

36 % and 19 %, respectively. The high degree of correlation between the increases in RNA, potassium and phosphorus is shown by the constant P/K^+, RNA/P and RNA/K^+ molar ratios (Figs. 1, 2); the more limited degree of correlation between magnesium content and the other three components is reflected in somewhat temperature-dependent K^+/Mg^{2+}, P/Mg^{2+} and RNA/Mg^{2+} molar ratios, also shown in these figures.
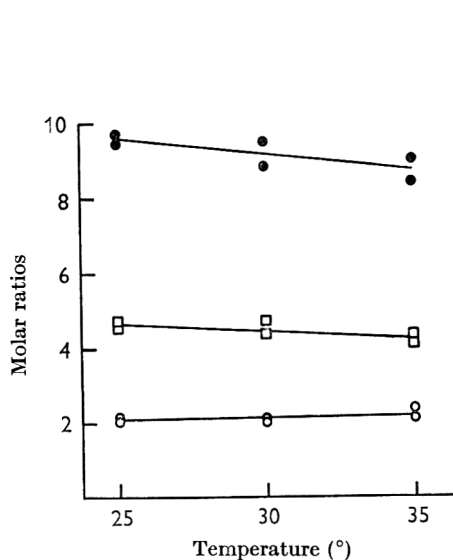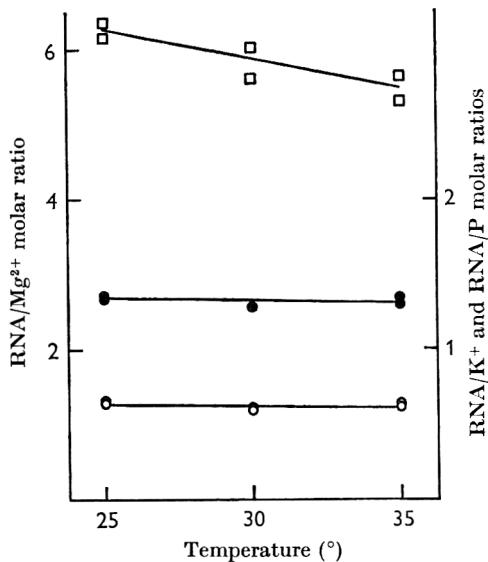
Fig. 1.   Fig. 2

Fig. 1. Stoichiometry between bacterial potassium, magnesium and phosphorus in K^+-limited chemostat cultures of *Aerobacter aerogenes* NCTC 418, grown at different temperatures. Molar ratios: ●, phosphorous: magnesium; □, potassium: magnesium; ○, phosphorus: potassium.

Fig. 2. Stoichiometry between bacterial RNA and potassium, magnesium and phosphorus in K^+-limited chemostat cultures of *Aerobacter aerogenes* NCTC 418, grown at different temperatures. For the purpose of calculating molar ratios, an average molecular weight for RNA-nucleotide of 340 was assumed. Molar ratios: □, RNA (nucleotide): magnesium; ●, RNA (nucelotide): potassium; ○, RNA (nucleotide): phosphorus.

Effect of growth rate on the steady state composition of Mg^{2+}-limited organisms growing at a fixed temperature

The results presented in Table 2 were obtained from an experiment similar to that from which the initial observations on the stoichiometry between intracellular potassium, magnesium, phosphorus and RNA were made (Tempest *et al.* 1966), but in which magnesium was made the growth-limiting component of the medium, potassium being supplied in excess of requirement. At dilution rates of 0·10 and 0·20 hr^{-1}, the K^+ concentration of the medium was set at 2 mM (1 mM-K_2SO_4), a proportion of which was not taken up by the organisms and could be measured in the culture supernantant fluid (see Methods). However, when the dilution rate was raised to 0·29 hr^{-1}, which imposed a growth rate well below the maximum usually observed for the organism growing in a simple salts medium, 'wash-out' occurred. This phenomenon was readily reversed by increasing the potassium concentration

Table 2. *Analysis of Mg^{2+}-limited Aerobacter aerogenes* NCTC *418*
grown at different dilution rates

Values quoted in this table were obtained from samples collected during 'steady state' growth at each dilution rate (D)

D (hr^{-1})	Bacterial concentration (equiv. mg. dry wt. bacteria/ml culture)	K$^+$	Mg^{2+} (g./100 g. dry bacteria)	P	RNA
0·10	5·56	0·89	0·115	1·60	9·7
0·20	3·96	1·14	0·136	1·74	11·1
0·29	3·04	1·14	0·158	1·89	12·1
0·48	2·67	1·52	0·192	2·28	15·4
0·68	2·44	1·67	0·201	2·34	16·8

of the medium to 4 mM, and 'steady-state' conditions were maintained on subsequent increases in dilution rate to 0·48 and 0·68 hr^{-1}.

The 73 % increase in RNA content of the organisms between growth rates of 0·10 and 0·68 hr^{-1} closely matched the increase in cellular magnesium (75 %) and potassium (88 %). The values shown in Figs. 3 and 4 make clear that the stoichiometry between RNA, potassium and magnesium remained independent of growth rate as the cellular content of individual components increased. The phosphorus content of the organisms also changed considerably with growth rate, increasing 46 % over the sevenfold increase in dilution rate. The degree to which the discrepancy between increase in cellular phosphorus and increase in potassium, magnesium and RNA contents affected the quantitative relationship between this
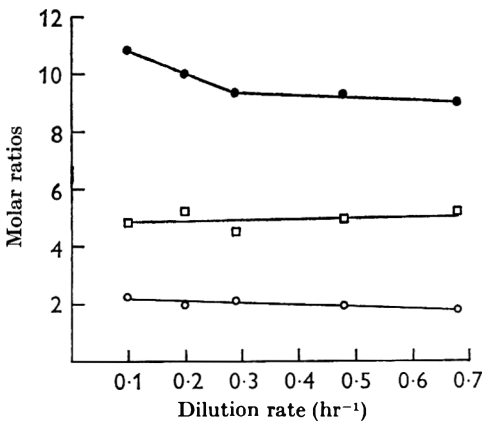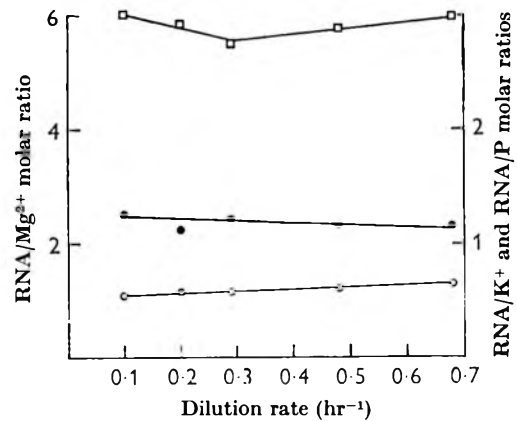
Fig. 3   Fig. 4

Fig. 3. Stoichiometry between bacterial potassiun, magnesium and phosphorus in Mg^{2+}-limited chemostat cultures of *Aerobacter aerogenes* NCTC 418, grown at different dilution rates. Molar ratios: ●, phosphorus:magnesium; □, potassium:magnesium; ○, phosphorus:potassium.

Fig. 4. Stoichiometry between bacterial RNA and potassium, magnesium and phosphorus in Mg^{2+}-limited chemostat cultures of *Aerobacter aerogenes* NCTC 418, grown at different dilution rates. For the purpose of calculating molar ratios, an average molecular weight for RNA-nucleotide of 340 was assumed. Molar ratios: □, RNA (nucleotide):magnesium; ●, RNA (nucleotide):potassium; ○, RNA (nucleotide):phosphorus.

component and the other three is seen in variations in the P/K^+, P/Mg^{2+} and RNA/P ratios (Figs. 3, 4). It is interesting to compare the phosphorus content of *Aerobacter aerogenes* as a function of growth rate under Mg^{2+}-limiting conditions in the present experiment with corresponding values for K^+-limited cultures (see Table 2 of Tempest *et al.* 1966). The value presented here for a dilution rate of 0.68 hr^{-1} (2.34% of dry weight) corresponds very closely with values of 2.44 and 2.39% at dilution rates of 0.69 and 0.70 hr^{-1}, respectively, for K^+-limited organisms. At a dilution rate of 0.10 hr^{-1}, however, the corresponding values are 1.60% (Mg^{2+}-limitation) and 1.31% (K^+-limitation). Thus, the partial breakdown in the stoichiometry between cellular phosphorus and the other components in Mg^{2+}-limited organisms seems to be the result of a relatively high phosphorus content of these organisms at low dilution rates.

Effect of growth rate on the steady-state composition of PO_4^{3-}-limited organisms growing at a fixed temperature

It is evident from Table 3 that the increases in cellular potassium, magnesium phosphorus and RNA for PO_4^{3-}-limited organisms over an eightfold increase in growth rate were greater than the corresponding increases in Mg^{2+}-limited (Table 2) and K^+-limited organisms (Tempest *et al.* 1966). Between dilution rates of 0.09 and 0.71 hr^{-1}, cellular RNA increased by over 270%, cellular magnesium by 200% cellular phosphorus by 190% and cellular potassium by nearly 150%. Under K^+-limitation (Tempest *et al.* 1966, their Table 1) and Mg^{2+}-limitation (Tempest *et al.* 1965, their Table 2), the carbohydrate content of the organisms was low, increasing in each case from about 2% of the dry weight at a dilution rate of 0.1 hr^{-1} to 4.5% at 0.8 hr^{-1}; PO_4^{3-}-limited organisms were different in that they contained 17–18% carbohydrate when grown at a dilution rate of 0.09 hr^{-1} (Table 3). Fractionation of these organisms showed that the bulk of this carbohydrate was glycogen. The carbohydrate content diminished as growth rate was increased, until at a dilution rate of 0.71 hr^{-1}, the value (4.4% of the dry weight) was very

Table 3. *Analysis of PO_4^{3-}-limited Aerobacter aerogenes NCTC 418 grown at different dilution rates*

The analyses were done on samples collected on different days during 'steady state' growth at each dilution rate (D).

D (hr⁻¹)	Bacterial concentration (equiv. mg. dry wt. bacteria/ ml. culture)	K⁺	Mg²⁺	P	RNA	Carbohydrate
				(g./100 g. dried bacteria)		
0·09	6·01	0·80	0·106	0·98	5·4	18·1
0·09	6·14	0·78	0·099	0·98	5·0	16·9
0·18	4·41	1·00	0·134	1·35	7·6	13·6
0·18	4·71	0·94	0·127	1·30	7·2	12·1
0·36	3·32	1·12	0·157	1·74	10·8	8·1
0·36	3·00	1·23	0·193	2·02	13·4	8·0
0·36	3·15	1·27	0·170	1·87	13·2	7·6
0·71	2·05	1·90	0·307	2·77	18·9	4·4
0·71	2·05	1·80	0·307	3·01	19·8	4·4

like that found for K^+-limited (4·7% at 0·8 hr^{-1}) and Mg^{2+}-limited organisms (4·29% at 0·8 hr^{-1}). The carbohydrate of PO_4^{3-}-limited *Aerobacter aerogenes* thus constituted a varying proportion of the bacterial dry weight as a function of growth rate, and the nature of the variation was such that it accentuated any increase in another cellular component with increasing growth rate. Therefore, the relatively great increases in the other four measured components in PO_4^{3-}-limited organisms compared with corresponding increases under conditions of Mg^{2+}- and K^+-limitation can be partially explained on these grounds.

The P/Mg^{2+} molar ratio did not vary significantly with growth rate (Fig. 5); the values reflected the high degree of correlation between the progressive increases in these two components as a function of dilution rate. The average value of these ratios (7·87) is close to the anticipated value of 8. Since cellular potassium increased somewhat less, and RNA rather more, than magnesium and phosphorus, molar ratios involving either RNA or potassium, and especially RNA/K^+, varied with growth rate (Fig. 6). However, examination of Figs. 5 and 6 indicates that the variation was essentially confined to dilution rates up to the value of 0·36 hr^{-1}. The molar ratios at 0·36 hr^{-1} were similar to those at the highest dilution rate used (0·71 hr^{-1}). This is particularly true for the P/K^+, RNA/P and RNA/K^+ molar ratios. When the molar ratios obtained at the two higher growth rates are averaged and expressed as a $Mg^{2+}:K^+:RNA$ (nucleotide):P ratio, the values obtained (1:4·09:4·89:8·02) are remarkably close to the predicted 1:4:5:8 ratio.
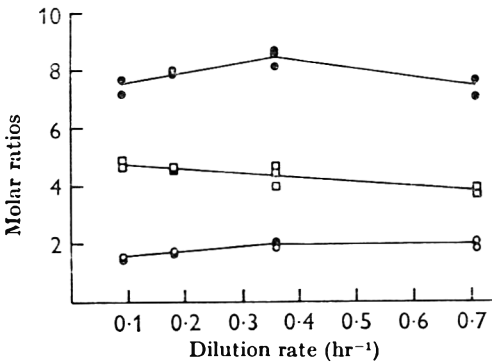
Fig. 5
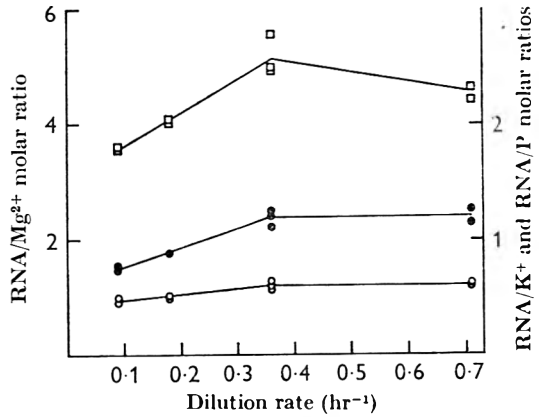
Fig. 6

Fig. 5. Stoichiometry between bacterial potassium, magnesium and phosphorus in PO_4^{3-}-limited chemostat cultures of *Aerobacter aerogenes* NCTC 418, grown at different dilution rates. Molar ratios: ●, phosphorus:magnesium; □, potassium:magnesium; ○, phosphorus:potassium.

Fig. 6. Stoichiometry between bacterial RNA and potassium, magnesium and phosphorus in PO_4^{3-}-limited chemostat cultures of *Aerobacter aerogenes* NCTC 418, grown at different dilution rates. For the purpose of calculating molar ratios, an average molecular weight for RNA-nucleotide of 340 was assumed. Molar ratios : □, RNA (nucleotide):magnesium; ●, RNA (nucleotide):potassium; ○, RNA (nucleotide):phosphorus.

DISCUSSION

The aim of the work described here was to measure the variation in potassium, magnesium and phosphorus contents of *Aerobacter aerogenes* NCTC 418 as a function of its RNA content. Two approaches were used. It is well established that the RNA content of micro-organisms increases with increase in growth rate (Herbert, 1961) and varying the growth rate under two separate growth limitations has been one of the techniques adopted to vary the bacterial RNA content. The second method is dependent on the observation of Tempest & Hunter (1965) that the RNA content of *A. aerogenes*, growing in a chemostat, varied with temperature at a fixed growth rate. Therefore, in a chemostat, the effect of temperature can be assessed independently of growth rate, which is controlled by a chosen dilution rate. Since the increase in cellular RNA observed by Tempest & Hunter (1965) as temperature was decreased at a fixed dilution rate, was ribosomal in origin, their results were interpreted in terms of a requirement for an increased number of ribosomes/cell to maintain the rate of protein synthesis imposed by the dilution rate under conditions where the activity of individual ribosomes was decreased by decreasing the temperature. This interpretation is consistent with the suggestion of Ecker & Schaechter (1963) that the rate of protein synthesis/ribosome particle is constant and independent of growth rate at any one temperature.

The high degree of correlation between the increase in RNA (presumably ribosomal), phosphorus and potassium contents of *Aerobacter aerogenes* as the temperature was decreased (Table 1) is considered to be evidence of an association between potassium and the ribosomal complement of these organisms. The observation that the quantitative relationship between RNA and magnesium is somewhat variable may be an indication that a significant proportion of the cellular magnesium is not bound to the ribosomes under K^+-limiting conditions at the low growth rate used. This suggestion might also explain the observation that the plot of RNA/Mg^{2+} molar ratio against dilution rate, described in a previous paper (Tempest *et al.* 1966, their fig. 5) exhibited a small positive slope, which can be interpreted as an indication that at higher growth rates, where the ribosome content of the organisms was increased, a larger proportion of the cellular magnesium was associated with the ribosomes.

The participation of phosphorylated intermediates in polysaccharide synthesis and the requirement of magnesium as a co-factor in many enzyme-catalysed phosphate transfer reactions are well established. The varying carbohydrate content of PO_4^{3-}-limited organisms may, therefore, not only account for the greater variation of magnesium and phosphorus contents of these organisms with growth rate relative to that found in K^+-limited and Mg^{2-}-limited organisms, but might explain also the low RNA/Mg^{2+} and RNA/P ratios at low growth rates (Fig. 6) on the basis that the synthetic reactions leading to polysaccharide demand significant amounts of magnesium and phosphate. Similarly, the participation of potassium in polysaccharide synthesis (Pulver & Verzar, 1940; Duguid & Wilkinson, 1954) may account for the low RNA/K^+ molar ratio at low growth rates. The observation that even the K^+/Mg^+ and P/K^+ ratios at dilution rates of 0·09 and 0·18 hr^{-1} were respectively higher and lower than predicted by the 1:4:8 stoichiometry between magnesium, potassium and phosphorus, suggests that the proportion of cellular potassium

associated with polysaccharide at low growth rates was relatively higher than the proportions of magnesium and phosphorus likewise involved. On this question, it is interesting to note that Poppen, Green & Wrenn (1953) found, by histochemical techniques, a correlation between the intracellular distribution of potassium and glycogen in heart and liver tissue.

The results presented in the present paper support the conclusions drawn previously (Tempest *et al.* 1966) that the major role of potassium in *Aerobacter aerogenes* (a role which demands the high intracellular concentration of this ion) is to be found in its association with the ribosomes. In the one case where a highly significant difference between the observed stoichiometry and that predicted was shown to occur, an explanation was put forward to account for the deviation. Thus, the relative excess of potassium over the other components of the relationship under PO_4^{3-}-limiting conditions at low dilution rates appears to be related to the accumulation of polysaccharide.

The possibility that potassium may be associated with some structural cellular component has, in the past, suffered from lack of convincing evidence in its favour, but recently this ion has been implicated in protein synthesis (Ennis & Lubin, 1961, 1965; Lubin, 1963). Lubin's conclusion that high concentrations of potassium are necessary for a 'priming reaction' which involves aminoacyl-S-RNA, messenger RNA, ribosomes and optimal concentrations of magnesium to yield an active complex for polypeptide synthesis is strengthened by evidence of Nakamoto *et al.* (1963) for the existence of such a complex. In cell-free systems, NH_4^+ appears to be two to three times more effective than K^+, but examination of the intracellular concentration of NH_4^+ favours the *in vivo* participation of K^+ (Lubin & Ennis, 1964). Another observation which appears to emphasize the specificity of potassium in protein synthesis was made by Lubin (1964), who pointed out that mature erythrocytes of some mammals contain low intracellular concentrations of potassium, but are rich in sodium; they do not synthesize protein.

Since at least three inorganic ions (K^+, Mg^{2+}, PO_4^{3-}) appear to be required in significant quantities for the synthesis and proper functioning of the protein-synthesizing apparatus of the cell, it is interesting to note that a close association between ion accumulation and growth in plants has been observed. Potassium, in particular, is located in regions of rapid growth, namely the apices and young leaves (Steele, 1949). Cation uptake associated with active growth (as distinct from vacuolar accumulation which also occurs in non-growing storage tissues) has been accounted for in terms of the neutralization of negative charges present on non-diffusible anions, which are synthesized during growth (Briggs, Hope & Robertson, 1961). Inasmuch as the nucleic acids of the ribosomes may account for a large proportion of such polyanions in the rapidly dividing cells of meristematic tissue, this explanation is consistent with the present proposition. Furthermore, Wood & Braun (1965) have shown that the rapidly growing cells of crown gall tumour tissue differ from cells of normal tissue in possessing more efficient mechanisms for uptake of potassium and phosphate, and higher concentrations of these cellular components. These authors believe that the transition from a normal to a fully autonomous tumour cell is dependent upon the development of an enhanced ability to absorb inorganic ions, higher intracellular concentrations of which result in the activation and loss of regulation of several growth-limiting synthetic processes.

REFERENCES

Blond, D. M. & Whittam, R. (1965). Effects of sodium and potassium ions on oxidative phosphorylation in relation to respiratory control by a cell-membrane adenosine triphosphatase. *Biochem. J.* 97, 523.

Briggs, G. E., Hope, A. B. & Robertson, R. N. (1961). *Electrolytes and Plant Cells.* Botanical Monographs. Ed. by W. O. James, vol. 1. Oxford: Blackwell Scientific Publications Ltd.

Dicks, J. W. & Tempest, D. W. (1966). Potassium metabolism in *Aerobacter aerogenes. Biochem. J.* 99, 7 p.

Dixon, M. & Webb, E. C. (1964). *Enzymes.* Second edition. London: Longmans Green and Co. Ltd.

Duguid, J. P. & Wilkinson, J. F. (1954). Note on the influence of potassium deficiency upon production of polysaccharide by *Aerobacter aerogenes. J. gen. Microbiol.* 11, 71.

Ecker, R. E. & Schaechter, M. (1963). Ribosome content and the rate of growth of *Salmonella typhimurium. Biochim. biophys. Acta* 76, 275.

Eddy, A. A. & Hinshelwood, C. N. (1951). Alkali-metal ions in the metabolism of *Bact. lactis aerogenes.* III. General discussion of their role and mode of action. *Proc. roy. Soc.* B 138, 237.

Ennis, H. L. & Lubin, M. (1961). Dissociation of ribonucleic acid and protein synthesis in bacteria deprived of potassium. *Biochim. biophys. Acta* 50, 399.

Ennis, H. L. & Lubin, M. (1965). Pre-ribosomal particles formed in potassium depleted cells. Studies on degradation and stabilisation. *Biochim. biophys. Acta* 95, 605.

Gardner, R. J. (1946). Colorimetric determination of magnesium in plasma or serum by means of Titan Yellow. *Biochem. J.* 40, 828.

Herbert, D. (1961). The chemical composition of micro-organisms as a function of their environment. *Symp. Soc. gen. Microbiol.* 11, 391.

Herbert, D., Phipps, P. J. & Tempest, D. W. (1965). The chemostat: design and instrumentation. *Lab. Practice* 14, 1150.

King, E. J. (1951). *Micro-analysis in Medical Biochemistry.* Second edition. London: Churchill Ltd.

Krebs, H. A., Whittam, R. & Hems, R. (1957). Potassium uptake by *Alcaligenes faecalis. Biochem. J.* 66, 53.

Lardy, H. (1951). The influence of inorganic ions on phosphorylation reactions. In *Phosphorus Metabolism.* Ed. by W. D. McElroy and B. Glass. Vol. i, p. 477. Baltimore: The Johns Hopkins Press.

Lester, G. (1958). Requirement for potassium by bacteria. *J. Bact.* 75, 426.

Lubin, M. (1963). A priming reaction in protein synthesis. *Biochim. biophys. Acta* 72, 345.

Lubin, M. (1964). Cell potassium and the regulation of protein synthesis. In *The Cellular Functions of Membrane Transport,* p. 193. New Jersey: Prentice-Hall Inc.

Lubin, M. & Ennis, H. L. (1964). On the role of intracellular potassium in protein synthesis. *Biochim. biophys. Acta* 80, 614.

Nakamoto, T., Conway, T. W., Allende, J. E., Spyrides, G. J. & Lipmann, F. (1963). Formation of peptide bonds. I. Peptide formation from aminoacyl-S-RNA. *Cold Spring Harb. Symp. quant. Biol.* 28, 227.

Partridge, S. M. (1949). Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* 164, 443.

Perry, J. J. & Evans, J. B. (1961). Role of potassium in the oxidative metabolism of *Micrococcus sodonensis. J. Bact.* 82, 551.

Poppen, K. J., Green, D. M. & Wrenn, H. T. (1953). The histochemical localization of potassium and glycogen. *J. Histochem. Cytochem.* 1, 160.

POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. *J. gen. Microbiol.* **34**, 459.

PULVER, R. & VERZAR, F. (1940). Connexion between carbohydrate and potassium metabolism in the yeast cell. *Nature, Lond.* **145**, 823.

ROBERTS, R. B., ROBERTS, Z. & COWIE, D. B. (1949). Potassium metabolism in *Escherichia coli*. 2. Metabolism in presence of carbohydrates and their derivatives. *J. cell. comp. Physiol.* **34**, 259.

ROUF, M. A. (1964). Spectrochemical analysis of inorganic elements in bacteria. *J. Bact.* **88**, 1545.

STEELE, C. C. (1949). An *Introduction to Plant Biochemistry*, p. 302. London: G. Bell and Sons Ltd.

STEINBACH, H. B. (1962). The prevalence of K. *Perspectives Biol. Med.* **5**, 338.

STRANGE, R. E., DARK, F. A. & NESS, A. G. (1961). The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. *J. gen. Microbiol.* **25**, 61.

TEMPEST, D. W. & HUNTER, J. R. (1965). The influence of temperature and pH value on the macromolecular composition of magnesium-limited and glycerol-limited *Aerobacter aerogenes* growing in a chemostat. *J. gen. Microbiol.* **41**, 267.

TEMPEST, D. W. & STRANGE, R. E. (1966). Variation in content and distribution of magnesium, and its influence on survival, in *Aerobacter aerogenes* grown in a chemostat. *J. gen. Microbiol.* **44**, 273.

TEMPEST, D. W., DICKS, J. W. & HUNTER, J. R. (1966). The interrelationship between potassium, magnesium and phosphorus in potassium-limited chemostat cultures of *Aerobacter aerogenes*. *J. gen. Microbiol.* **45**, 135.

TEMPEST, D. W., HUNTER, J. R. & SYKES, J. (1965). Magnesium-limited growth of *Aerobacter aerogenes* in a ehemostat. *J. gen. Microbiol.* **39**, 355.

TREVELYAN, W. E., PROCTOR, D. P. & HARRISON, J. S. (1950). Detection of sugar on paper chromatograms. *Nature, Lond.* **166**, 444.

WOOD, H, N. & BRAUN, A. C. (1965). Studies on the net uptake of solutes by normal and crown gall tumor cells. *Proc. natn. Acad. Sci. U.S.A.* **54**, 1532.

Books Received

Acta Microbiologica (Academiae Scientiarum Hungaricae). Edited by G. IVANOVICS. Published by Akademiai Kiado, Budapest. 187 pp.

Annual Review of Microbiology, Vol. 20, 1966. Edited by C. E. CLIFTON. Published by Annual Review Inc., California, U.S.A. 457 pp. Price $9.00.

Biology of Parasites (Ephasis on Veterinary Parasites). Edited by E. J. L. SOULSBY. Published by Academic Press, New York and London. 354 pp. Price $13.50.

Contributions to Helminthology: from the Academy of Sciences of the U.S.S.R. Translated from Russian by the Israel Program for Scientific Translations, Jerusalem. Published by Oldbourne Press, London, E.C. 1.

Index Bergeyana. Edited by R. E. BUCHANAN, J. G. HOLT and E. F. LESSEL, JUN. Published by E. and S. Livingstone Ltd., Edinburgh and London. 1472 pp. Price £10.

Keys to Actinomycetales. By N. A. KRASI'NIKOV. Published by the Israel Program for Scientific Translations. Translated from Russian. Distributed by Oldbourne Press, London. 168 pp. Price £2. 5s.

Molecules and Evolution. By THOMAS H. JUKES. Published by Columbia University Press, London. 282 pp. Price 72s.

Principles of Microbial Ecology. By T. D. BROCK. Published by Prentice-Hall, Englewood Cliffs, New Jersey, U.S.A. and London. 525 pp. Price $7.75 or 62s.

Protein Biosynthesis and Problems of Heredity Development and Ageing. By ZHORES A. MEDVEDEV (U.S.S.R.). Translated by ANN SYNGE. Published by Oliver and Boyd, Edinburgh and London. 558 pp. £5. 10s.

Revista Venezolana de Sanidad Y Asistencia Social. Vol. xxx. Published by Binlioteca a/c Institutuo Nacional de Higiene, Apdo 5153, Correos del Este, Avda. Miranda, Caracas, Venesuela, S.A. 123 pp.

The Rumen and its Microbes. By ROBERT E. HUNGATE. Published by Academic Press Inc. New York and London. 525 pp. Price $19.75.

The Therapeutic Use of Antibiotics in Hospital Practice (Proceedings of a Symposium Held at St Thomas's Hospital Medical School). Edited by M. RIDLEY and I. PHILLIPS. Published by E. and S. Livingstone Ltd. Edinburgh and London. 223 pp. Price 25s.

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-seventh General Meeting at The Medical School, University of Bristol, on Tuesday, Wednesday and Thursday 27, 28 and 29 September 1966. The following communications were made:

ORIGINAL PAPERS

Enzymes of Acetate Metabolism in Blue-green Algae. By J. Pearce and N. G. Carr (*Department of Biochemistry, University of Liverpool, Liverpool 3*)

The photo-assimilation of acetate by *Anacystis nidulans* and the presence of acetyl-CoA synthetase has been demonstrated (Hoare, D. A. & Moore, R. B. (1965), *Biochim. Biophys. Acta* 109, 622). In a previous communication we have reported the incorporation of [2^{14}C]acetate into the cell material of two species of blue-green algae, but the failure of acetate and other organic substrates to stimulate growth was confirmed (Carr, N. G. & Pearce, J. (1966), *Biochem. J.* 99, 28 P). Activities of some enzymes associated with acetate metabolism have now been measured in cell free extracts of *A. nidulans* and *Anabaena variabilis*. The latter organism differed from the former in possessing acetate kinase [EC 2.7.2.1] rather than acetyl-CoA synthetase [EC 6.2.1.1]; the levels of neither of these enzymes were altered after growth in the presence of acetate. The enzymes of the glyoxylate cycle, known to be operative and under adaptive control in a wide range of micro-organisms (Kornberg, H. L. & Elsden, S. R. (1961), *Adv. Enzymol.* 23, 401) have been demonstrated in these algae. The measurement of isocitrate lyase [EC 4.1.3.1] was complicated by a high level of isocitrate dehydrogenase [EC 1.1.1.42], this difficulty being surmounted by the separation of enzymic activities by centrifugation at 78,000 g for 20 hr. The isocitrate lyase remained soluble whilst the isocitrate dehydrogenase sedimented. The specific activity of these two enzymes, as well as those of malate synthetase [EC 4.1.3.2] and citrate synthatase [EC 4.1.3.7] did not vary significantly between extracts of organisms grown in the presence or absence of acetate.

These results indicate that the two blue-green algae examined possess enzymes necessary for the activation of acetate and its entry into the glyoxylate and tricarboxylic acid cycles. However, these algae do not exhibit adaptive control of enzyme activity and this failure to adjust enzymic activity to environmental change may account for the lack of response in growth rate and respiration when organic substrates are supplied.

Microbial Metabolism of 1-Aminopropan-2-ol. By M. A. Pickard, I. J. Higgins and J. M. Turner (*Department of Biochemistry, University of Liverpool*)

A variety of micro-organisms oxidize aminopropanol to aminoacetone (Turner, J. M. (1966), *Biochem. J.* 98, 7 P), and microbial growth on aminopropanol (Gottlieb, S. F. & Mandel, M. (1959), *Can. J. Microbiol.* 5, 363; Higgins, I. J. & Turner, J. M. (1966), *Biochem. J.* 99, 26 P) offers the opportunity of studying the metabolism of the amino ketone. A pseudomonad, capable of growth on either aminopropanol or aminoacetone, has been used in such studies. An L(+)-1-aminopropan-2-ol—NAD/NADP oxidoreductase has been purified 50-fold from extracts of the micro-organism grown on aminopropanol. A 70-fold increase in extract activity was effected by incubating cells with aminopropanol, and analogues except for L-threonine were ineffective as inducers. The enzyme has optimum activity at pH 9·6, requires NAD or NADP as coenzyme, has activity unaffected by sulphydryl or chelating reagents, is readily reversible, and is stereospecific for L(+)-

a

aminopropanol. Virtually no activity was found with eighteen substrate analogues, with the exception of DL-2-hydroxy-2-phenylethylamine and 2-aminoacetophenone which were readily oxidized and reduced respectively. The pH optimum for aminoacetone and 2-amino-acetophenone reduction was approx. 5·0, and the reaction was inhibited by phosphate. Kinetic constants for the enzyme (molecular weight 70–80,000), have been determined. The further metabolism of aminoacetone has been investigated using both intact micro-organisms and cell-free preparations.

Control of Constitutive β-Galactosidase Synthesis in *Escherichia coli*. By W. H. HOLMS (*Department of Biochemistry, University of Glasgow, Glasgow*)

Constitutive enzyme synthesis has not received as much attention as the phenomenon of inducibility (A. B. Pardee & J. R. Beckwith (1963), in *Informational Macromolecules*, New York and London: Academic Press). Most work has been done with constitutive mutants, such as the strain (ATCC 15224, ML 308, $i^-z^+y^+$) used in this work which was originally isolated at the Institut Pasteur. Mandelstam ((1962), *Biochem. J.* 82, 489) has shown that the specific activity of β-galactosidase in this strain can be varied by changes of nutritional limitations and flow rates in a chemostat. The purpose of the present report is to describe some aspects of the control of β-galactosidase synthesis in batch cultures.

Cultures grown in glucose–salts and stored overnight at 4°, had only 24·7 % of the specific activity of the enzyme as had cultures grown similarly in glycerol–salts. Cells of high and low specific activity were prepared in this way, washed in growth medium without energy source, and used as inocula for ammonia–salts media with glucose, glycerol or lactose as energy source. When cells of low specific activity were tested there was a more rapid exponential increase of β-galactosidase than turbidity in all media, but the differential rate of enzyme synthesis was greatest in glycerol. When cells of high specific activity were used as inoculum different results were obtained. In glucose medium, enzyme doubled somewhat more slowly than turbidity and in glycerol medium somewhat faster. Surprisingly, there was a long lag before growth commenced in lactose medium, when enzyme synthesis was equal to or slower than growth.

Differences in doubling time of enzyme and turbidity were reflected in large changes of the specific activity of β-galactosidase. These control mechanisms apparently responded both to change in environment and to enzyme level in the inoculum.

Periodic Changes in Amidase Synthesis by *Pseudomonas aeruginosa* in Continuous Culture. By A. BODDY, P. H. CLARKE (*Department of Biochemistry, University College, London*) and M. D. LILLY (*Biochemical Engineering Section, Department of Chemical Engineering, University College, London*)

Pseudomonas aeruginosa 8602 produces an aliphatic amidase which is induced by acetamide and a few other amides and is subject to catabolite repression by succinate (Brammar, W. J. & Clarke, P. H. (1964), *J. gen. Microbiol.* 37, 307).

The culture was grown to a constant bacterial concentration in continuous culture in a 3-litre vessel with 10^{-2} M succinate as carbon source. The changes in bacterial concentration and the kinetics of amidase synthesis were studied when the ingoing medium was changed to 10^{-2} M succinate $+ 2 \times 10^{-2}$ M acetamide at different flow rates.

At low dilution rates ($D = 0.2$) the bacterial concentration did not increase smoothly but fluctuated about the predicted value. With little lag the amidase activity rose rapidly fluctuating with a periodicity of about 1 hr. Further measurements were made 24 hr. after the change to succinate + acetamide medium and these oscillations were still occurring.

At higher dilution rates (e.g. $D = 0.5$) there was marked catabolite repression of amidase synthesis. Sufficient amidase was produced soon after the change to succinate + acetamide medium to allow utilization of the acetamide and a corresponding increase in the bacterial concentration. The amidase level, however, remained much lower than that observed for lower dilution rates and only began to rise rapidly several hours after acet-

amide was introduced to the system. Again, periodic fluctuations were observed in bacterial concentration and enzyme level and these oscillations were still present after 24 hr.

These results showing undamped oscillations of an inducible enzyme in continuous culture even under 'steady state' conditions are in accord with the theoretical predictions made by Goodwin, B. C. ((1966), *Nature, Lond.* 209, 479). The marked difference in catabolite repression of amidase synthesis at slow and fast flow rates clearly reflects the relative rates of intracellular utilization of succinate.

The Growth of Animal Cells in Suspension Cultures at Controlled Oxygen Tensions. By D. G. KILBURN and F. C. WEBB (*Biochemical Engineering Section, Department of Chemical Engineering, University College, London*)

The O_2 tension for optimum growth of suspension cultures of animal cells has been observed to be less than atmospheric (Cooper, P. D., Wilson, J. N. & Burt, A. M. (1959), *J. gen. Microbiol.* 21, 702; Brosemer, R. W. & Rutter, W. J. (1961), *Expl Cell Res.* 25, 101). Definite confirmation of this finding requires the measurement of O_2 tension in the liquid phase and the control of this variable during growth.

A 5-l Porton-type fermentor was adapted so that the effect of oxygen tension on the growth of mouse LS cells could be studied in a controlled environment. The effluent gas from the vessel was analysed for O_2 and CO_2 and the pH was controlled by alkali addition. The dissolved O_2 tension was measured using a modified Mackereth electrode and controlled by sparging air once per min. if the measured value dropped below the set point. A continuous oxygen demand (even in the absence of organisms) was maintained by flushing the head space of the vessel with N_2.

In experiments without air sparging and the suspension occupying 50 % of the vessel volume, with a flow of air through the head space, it was found that dissolved O_2 tension decreased linearly to a low value (less than 2 mm. Hg); this was maintained during the latter part of the exponential growth phase.

At controlled O_2 tension levels, present results indicate that the optimum tension for growth of the LS cell is about 80 mm. Hg. This shows up particularly clearly under continuous conditions.

Heterotrophic Uptake of Low Organic Solute Concentrations by Marine Micro-organisms. By A. L. S. MUNRO (*Marine Laboratory, Department of Agriculture and Fisheries for Scotland, Aberdeen*)

The incorporation of several ^{14}C labelled organic substrates into naturally occurring planktonic and benthic micro-organisms has been demonstrated. The velocity of uptake into cell material was measured over the substrate range 0·002–6·0 mg./l. The shape of the curve representing velocity of uptake against concentration was found in many, but not all, cases to be similar to the results of velocity of uptake of glucose and acetate described by Wright & Hobbie ((1965), *Limnol. Oceanog.* 10, 22) for fresh-water planktonic micro-organisms. These latter authors have described how two separate and competing mechanisms of uptake can be differentiated from the velocity curves by using laboratory cultures of planktonic bacteria and algae. Specific transport mechanisms effective at low substrate concentrations were traced to bacteria and a diffusion mechanism, effective only at higher concentrations, to the algae.

Autoradiographic techniques (Brock, T. D. & Brock, M. L. (1966), *Nature, Lond.* 209, 734) using ^3H glucose and acetate as substrate have not confirmed any participation in the incorporation of substrate by the numerous diatoms present at any concentration of substrate tested. The majority of areas of grain development cover 1–3 μ and in rare cases very small cocco-bacillary organisms can be seen. No areas of grain development occur in zero time formalin killed controls. Some preparations contain a small number of unidentified unicellular organisms, 3–15 μ in size and are possibly small flagellates, which show dense areas of grain development at *all* concentrations so far tested.

The results of autoradiography show that only a small minority of the algae present

participate in heterotrophy even at high solute concentrations (5 mg./l.) where Wright & Hobbie predicted considerable uptake by algae. Therefore other explanations for the shape of the velocity of uptake curve, such as physiological state or species composition of the microbial population, must be sought.

Properties of Mitochondria from *Saccharomyces carlsbergensis* Grown in Continuous Culture. By P. Ann Light and P. B. Garland (*Biochemistry Department, University of Bristol*)

The recent development of techniques for the preparation of yeast mitochondria with relatively intact energy conservation mechanisms as employed by Duell, Inoue & Utter ((1964), *J. Bacteriol.* 88, 1762) and Ohnishi, Kawaguchi & Hagihara ((1966), *J. biol. Chem.* (in Press)) leads to the prospect of using microbiological approaches for mitochondrial problems. In view of the advantages of continuous culture over batch culture we have investigated the production of mitochondria from *Saccharomyces carlsbergensis* (74S N.C.Y.C.) grown in a 0·5 litre working volume Porton type chemostat (Herbert, D., Elsworth, R. & Telling, R. C. (1956), *J. gen. Microbiol.* 14, 601). Aerobic growth at 30° on DL-lactate as carbon source at a dilution rate of 0·3 hr^{-1} yielded 15–20 g. wet weight of cells in an overnight (12 hr) collection, and approximately 200–300 mg. mitochondrial protein were obtained by the procedure of Duell, Inoue & Utter (1964) using snail gut enzyme and 2-mercaptoethylamine. Oxidation rates, respiratory control and P/O ratios measured polarographically were strictly comparable with those described by Onishi, Kawaguchi & Hagihara (1966). Aerobic growth on glucose gave lower yields of mitochondria. Measurements in cell extracts of acetylcarnitine transferase activity (E.C. 2.3.1.7), which is a membrane bound mitochondrial enzyme, indicated that mitochondrial synthesis was partially repressed with glucose as carbon source.

Effect of Polysorbate 80 on Cell Leakage of *Pseudomonas aeruginosa*. By M. R. W. Brown and B. E. Winsley (*Pharmaceutical Microbiology Group, School of Pharmacy, College of Science and Technology, Bristol*)

Previous work has shown that polysorbate 80 enhanced the inhibition of *Pseudomonas aeruginosa* by various chemical agents (Brown, M. R. W. & Richards, R. M. E. (1964), *J. Pharm. Pharmac. Suppl.* 16, 51T). The effect of polysorbate on permeability of this organism was investigated by measuring the leakage of substances with a maximum absorption at 260 mμ. (Salton, M. R. J. (1951), *J. gen. Microbiol.* 5, 391). Cultures of *P. aeruginosa* NCTC 6570 were grown in nutrient broth containing graded concentrations of polysorbate. Washed cells were finally resuspended in distilled water at a concentration of 10^9/ml. Samples from these stored suspensions were centrifuged and the optical density of the supernatant measured at 260 mμ. Increasing concentrations of polysorbate in the growth medium resulted in increasing leakage from the washed suspensions up to a maximum at about 0·13 % polysorbate. At concentrations higher than this there was reduced leakage which was, nevertheless, greater than that in the control suspension of cells grown in the absence of polysorbate. This pattern of leakage was the same at 37°, 18° and 4°. The greatest amount of leakage occurred at 37° and the least at 18°. When the distilled water in the final suspensions was replaced by solutions of various concentrations of sodium chloride, the leakage decreased with increasing amounts of sodium chloride up to a concentration of 2M, the highest used. Similar permeability experiments were performed using cells grown in the absence of polysorbate but finally suspended in solutions of polysorbate in water; optical density readings at 260 mμ were corrected for absorption by polysorbate. Substantially the same pattern of leakage occurred with a maximum at about 0·13 % polysorbate. These results suggest that active cell growth is not necessary for polysorbate to produce its effects on permeability. The contribution of cell lysis to absorption was assessed by making total (chamber) counts during the course of the permeability experiments. The results indicate that a small but significant amount of lysis contributed to the amount of substance absorbing at 260 mμ.

The Presence of Hyaluronic Acid in the Extracellular Slime of *Pseudomonas aeruginosa*. By M. R. W. Brown, J. R. Clamp and J. H. S. Foster (*Pharmaceutical Microbiology Group, School of Pharmacy, College of Science and Technology, Bristol, and Department of Medicine, Bristol Royal Infirmary, Bristol*)

Pseudomonas aeruginosa produces extracellular slime when grown in static culture using a variety of carbon sources (Haynes, W. C. (1951), *J. gen. Microbiol.* 5, 930). The major constituent has been identified as hyaluronic acid (Warren, G. H. & Gray, J. (1954), *J. Bact.* 67, 167; Bonde, G. J., Carlsen, F. E. & Jensen, C. E. (1957), *Acta pharmac. tox.* 13, 205). It has also been reported that the major component is mannan, with small amounts of DNA, RNA and no detectable uronic acid (Eagon, R. G. (1956), *Can. J. Microbiol.* 2, 673; (1962) *ibid.* 8, 585). Static cultures of several strains of *P. aeruginosa* were grown in nutrient broth for 5 days. The viscous cultures were then homogenized for 30 sec. in a Waring blendor and the cells removed by centrifugation. The slime was precipitated by 1 volume of ethanol, redissolved in warm water and reprecipitated with ethanol. The slime was washed with ether and dried over P_2O_5 *in vacuo*. The slime was hydrolysed (Clamp, J. R. & Putnam, F. W. (1964), *J. biol. Chem.* 239, 3233), and after concentration subjected to descending chromatography for 18 hr in butanol/ethanol/water (4:1:2) + 1 % ammonia. The sugars were visualized with *o*-aminodiphenyl reagent. Spots corresponding to mannose, glucose, glucuronic acid, ribose and traces of rhamnose were indicated. When the chromatogram was stained with ninhydrin, glucosamine was indicated. The presence of glucuronic acid and glucosamine may be explained on the basis of hyaluronic acid hydrolysis. The sizes of the spots suggested that there was about 4 times as much glucose as mannose.

Hyaluronic acid was also shown in the unhydrolysed slime by zone electrophoresis on cellulose acetate, after staining with alcian blue. Infrared spectra of the slime also clearly indicated the presence of hyaluronic acid in the slime (Orr, S. F. D. (1954), *Biochim. biophys. Acta* 14, 173). Quantitative estimation of glucuronic acid (Bitter, T. & Muir, H. M. (1962), *Anal. Biochem.* 4, 330) and glucosamine (Gatt, R. & Berman, E. R. (1966), *Anal. Biochem.* 15, 167) consistently indicated a content of about 3–5 % hyaluronic acid in the slime.

Bacteria Counting with a Coulter Counter. By P. N. Hobson, S. O. Mann and R. Summers (*The Rowett Research Institute, Bucksburn, Aberdeen*)

We have used a Counter (Coulter Electronics Ltd., Dunstable, Bedfordshire) for counting bacteria in various experiments. Saline for dilutions was a problem; it was impossible to produce low-particle saline by laboratory filtration, but sterile saline for injection has usually a sufficiently low count. In most counts a 30 μ orifice tube with a gain setting of 6 and an aperture current setting of 8 have been used. Difficulties have been found in dilutions of broth cultures because of blocking of the orifice with particles from Seitz filter-sterilized medium constituents. No trouble from this was found with samples from continuous cultures where the large volumes of medium could settle for some days before flowing into the culture. Reasonable linear correlations between counts and turbidity of batch cultures have been found for *Selenomonas ruminantium* and *Peptostreptococcus elsdenii*. A *Veillonella* species gave completely erratic counts, probably due to the small size of the cells and the formation of clumps. A suspension of *Ruminococcus albus* cells diluted to different extents gave counts proportional to the dilutions. Suspensions of *S. ruminantium* cells used for survival experiments could rapidly be standardized with the Counter. Counts of lipolytic bacterium 5S and *Bacteroides amylophilus* from continuous cultures gave linear relationships with cell dry weight and turbidity of the cultures (at different growth rates). From these relationships maximum growth rates could be obtained by measuring turbidities at different stages of growth in batch cultures, and these corresponded with rates found in continuous cultures. By light centrifuging to remove large debris and protozoa before dilution in saline it was possible to obtain counts of the bacteria in rumen contents of animals on a number of diets. These counts agreed with counts made

using a counting chamber, and in eighteen samples from six sheep on a semisynthetic diet the total count generally reflected the viable count. Also the occasional presence of a large unculturable organism was detected on the Counter and counts were made of this.

On the Variability of Counts of Bacterial Colonies. By G. J. BONDE (*Institute of Hygiene, University of Aarhus, Denmark*)

Colony counts (viable counts) are often used for the estimation of bacterial numbers in the grading of water, milk, urine, etc. Computations of mean and standard deviation, and tests of significance are very often performed without taking into consideration that such operations presuppose a knowledge of the underlying distribution function of the data.

Viable and total counts have since the early papers by 'Student' and by Fisher *et al.* ((1922), *Ann. appl. Biol.* 9, 325) been supposed to obey the Poisson law. However, some authors have called attention to the fact that far greater variability could be demonstrated with biological data (Armitage, P. (1957), *J. Hyg. Camb.* 55, 564; Bonde, G. J. (1962), *Bacterial Indicators of Water Pollution,* Tekn. Forl.). In the latter monograph Bonde demonstrated from 7080 counts of *Clostridium perfringens* in 707 samples of water, sewage, and sludges, that only within a smaller group of data was the Poisson law obeyed, and no data followed the Polya distribution.

Extended experiments comprising a further 5820 counts of *Clostridium perfringens* in 582 samples of sludge, and 1652 counts of pure culture suspensions of *Escherichia coli* has thrown light on some unexplained details and further confirmed that for a description of the relations between variance and mean the following general model could be applied

$$\sigma^2 \simeq \gamma \, \mu^\beta.$$

γ depended upon the nature of the material and was estimated at 0–1·5. β was estimated at 1·12; it, presumably, depended more upon the treatment of the material, and was, for instance, of different value after heat treatment of the sample. This type of distribution is supposed to be provoked by a splitting of clumps and chains in the sample, whereas a Polya distribution is caused by a more thorough inhomogeneity of material or procedure. Justification for these assumptions was provided by experiments involving controlled clump splitting and inhomogeneity; and by microphotographs which suggest that inoculation of suspensions containing only free floating single bacteria is a rare occurrence.

Application of normal distribution (or of square-root transformations) would result in too small variances and misleading results of tests of significance.

Measurement of Yields from Cultures Growing on Synthetic Organic Compounds. By W. J. PAYNE, G. J. PROCHAZKA and W. R. MAYBERRY (*Department of Bacteriology, University of Georgia, Athens, Georgia, U.S.A.*)

Studies of bacterial dissimilation of synthetic organic compounds in our laboratory have revealed several previously undescribed enzymes (i.e. primary alcohol sulphatase, secondary alcohol sulphatase, ether glycol dehydrogenase) which we have reported (Payne, Williams & Mayberry (1965), *Appl. Microbiol.* 13, 698; Payne & Todd (1966), *J. Bact.* 91, 1533)). We have demonstrated, moreover, the reliability of bacterial growth as an indicator of the degree of degradation of synthetic organic compounds (Prochazka & Payne (1965), *Appl. Microbiol.* 13, 702); and we are now engaged in a study of parameters associated with growth at the expense of synthetic organic compounds to determine their expected input energy for ecosystems into which they may be released. Measurement of cell yields from the water-insoluble compound, dodecanol, provided scattered data when either dry cell weight or optical density of cultures was employed. Determination of the ratio of oxygen consumed to substrate utilized is a more dependable parameter, but even so smoothly correlated data are not always obtained. Gas-liquid chromatography has been indispensible

to the study of dissimilation of dodecanol, for most other analytical techniques are too insensitive or imprecise.

Measurement of yields by dry cell weight and turbidity of cultures provided closely correlated data when bacteria were grown on the water-soluble compounds, di-, tri-, and tetraethylene glycols, benzoic acid and phenylacetic acid. The ratio of quantity of oxygen consumed to substrate utilized was found to be a very reproducible datum with these substrates. The gas–liquid chromatograph was again most valuable for measuring metabolites, and a column system for the determination of the ether glycols was devised. Calculations based on possible pathways of dissimilation, degree of reduction of substrate and quantity of substrate diverted to construction have been made to attempt to correlate our estimates of energy yielded with those of others (Bauchop & Elsden (1960), *J. gen. Microbiol.* **23**, 457).

The Effect of Growth Rate on the Maintenance of Penicillin Synthesis and Morphology of *Penicillium chrysogenum*. By S. J. PIRT and R. C. RIGHELATO (*Microbiology Department, Queen Elizabeth College, University of London, W.* 8)

Continuous cultivation of *Penicillium chrysogenum* WIS 54-1255 under glucose-limited growth conditions showed the independence of the specific penicillin production rate q_{pen} (units/mg. dry weight/hr) and specific growth rate over the range $0 \cdot 027 - 0 \cdot 086$ hr^{-1}. The maximum growth rate was $0 \cdot 095$ hr^{-1}. This independence does not apply at much lower growth rates. In two stage continuous processes with little or no growth in the second stage, the second stage q_{pen} will be determined by the rate of decay of the penicillin synthetic system. Pirt ((1966), in *Microbial Physiology and Continuous Culture*, London: H.M.S.O., in Press) has shown how the production in the second stage can be calculated provided that the decay rate is known.

To investigate the maintenance of the penicillin-producing ability at a growth rate of zero, the medium flow to a single-stage continuous culture was stopped and only phenylacetic acid and glucose fed, the latter at the rate calculated from growing cultures to be the maintenance requirement for glucose (Pirt, S. J. (1965), *Proc. roy. Soc.* B **163**, 224).

The dry weight, oxygen consumption and carbon dioxide output were maintained constant for up to 200 hr. The q_{pen} fell approximately linearly with time to zero. The time taken to reach zero varied as a function of the growth rate in the continuous growth phase. With the highest growth rate the rate of decay was at a minimum (*c.* $0 \cdot 6 \%$/hr); with the lowest growth rate the q_{pen} fell at *c.* 2%/hr.

In continuous growth the organism produced long thin hyphae. During the non-growing phase these broke into shorter lengths and finally formed spores as the q_{pen} approached zero. Another strain, which normally was slow to sporulate on agar media, did not sporulate in the submerged cultures during the non-growing period, but the decay rate of the q_{pen} ($\%$/hr) was similar to that of strain 54-1255.

Investigation into the Relation between the Average Surface Area and the Average Number of Receptors for Colicine E2 of Bacteria from Cultures of Different Ages. By C. SHIMELD (*Department of Bacteriology, University of Bristol, Bristol* 1)

It has been known for some time that the sensitivity of bacterial cells to such agents as ultraviolet light (Stapleton, G. E. (1955), *Bact. Rev.* **19**, 26) and antibiotics (Quesnel, University of Bristol Ph.D. Thesis) varies with the age of the cell. With *Escherichia coli* C6 and colicine E2, which is produced by *Shigella sonnei* P/9/0, early log phase cultures appeared to be far more sensitive than stationary phase cultures. Adsorption experiments were carried out to determine the average number of receptors possessed by *E. coli* C6 for this colicine and this number was found to vary according to the age of the culture from which the adsorbing cells were obtained. Cells from young cultures have on the average more receptors than those taken from old cultures which have reached the stationary phase and are therefore more sensitive to colicine.

Measurements of the length and width of stained preparations of cells from different-aged

cultures have been made using an image splitting eyepiece and from these measurements the average surface area can be calculated. It was found that cells from log phase had a larger average surface area than those from the stationary phase and this surface area could be correlated with their number of receptors and hence their sensitivity to colicine.

Acridines and the Vegetative Reproduction of Bacteriophage λ. By ANGELA BAILEY, M. IOSSON and B. A. FRY (*Department of Microbiology, University of Sheffield*)

The vegetative reproduction of bacteriophage λ, a temperate phage, was more sensitive to inhibition by proflavin, 5-aminoacridine (5AA) and acridine than growth of the host, *Escherichia coli* strain K112. Concentrations of acridines which had a small or no effect on growth, markedly reduced phage λ reproduction, but in contrast to their effect in the virulent T phage system (Susman, M., Piechowski, M. M. & Ritchie, D. A. (1965), *Virology*, **26**, 163) inhibition was not complete. In such conditions, the average burst size was 20 % of the control: there was no effect on the time at which the phage infected bacteria lysed. Single burst experiments showed that presence of the acridine reduced the number of bacteria which released active phage and that the bacteria which did yield phage produced a much smaller number of particles/bacterium. In the conditions used the latent period was 38 min. If the acridine was withdrawn before 20–25 min. after infection, the average burst size was normal. If the acridine was added at progressively later times in the latent period, then from 20 min. onwards it was progressively less inhibitory. Such results could mean that as in the T phage system (Foster, R. A. C. (1948), *J. Bact.* **56**, 795; Susman *et al.* 1965) the acridines affect a late stage in the assembly of complete phage particles from previously synthesized precursors. However, this may not be the only or correct explanation of the inhibitory action of acridines in the temperate phage system. Thus in the presence of proflavin, bacteria infected with phage λ synthesized an amount of infective phage λ DNA which was of the same order as the reduced phage yield. This contrasts with the *E. coli*-T_4 system where 5AA had no such inhibitory effect on phage DNA synthesis (Susman *et al.* (1965)).

Rubella Virus and the Plaque Test in RK₁₃ Cultures. By M. BUTLER (*Department of Virology, Wellcome Research Laboratories, Beckenham, Kent*)

RK$_{13}$ cultures (Beale, A. J., Christofinis, G. C. & Furminger, I. G. S. (1963), *Lancet* **2** 640) showed a variable capacity to support plaque formation in plates by rubella virus, Plaque morphology was influenced by the depth of the overlay and certain medium variables such as the concentration and batch of agar, the batch and species of serum, and the concentration of sodium bicarbonate. In effect, virus adsorption was complete within 5–10 min. although at this time some of the virus could be recovered by washing the cell sheet. After 2 hr adsorption no virus was removed, but by this time only half the plaque forming units developed. Consideration of these factors made it possible to select optimal conditions for the test which proved of value in viral studies.

Using the plaque test it was possible to differentiate by size between rubella virus strains. Furthermore, plaque size varied according to the previous passage history of the virus, thus passage in RK$_{13}$ cultures increased plaque size, whereas passage in dog, calf, rabbit and monkey kidney tissue had no such effect. Passage in human embryo lung diploid cell strain appeared to result in the loss of plaque forming ability.

The plaque reduction technique was a more sensitive appraisal of the presence of antibody than the indirect test in tube cultures (Parkman, P. D., Beuscher, E. L. & Arterstein, M. S. (1962), *Proc. Soc. exp. Biol. Med.* **111**, 225), since definite values could be allotted to sera with very low neutralization titres. In general, however, 50 % plaque reduction gave a neutralization index about the same as the indirect test in which 50 % neutralization of tissue culture dose was measured.

Infective dose measured by the plaque test was slightly higher than the titre measured in the indirect tube test.

SYMPOSIUM: THE MEANING OF MEASUREMENT IN STUDIES OF GROWTH AND INHIBITION OF BACTERIA

The Significance of Variations in Bacterial Concentration, Biomass, Yield and Viability in Continuous Cultures of *Aerobacter aerogenes*. By D. W. TEMPEST (*Microbiological Research Establishment, Porton Down, Wiltshire*)

Micro-organisms can be grown in the chemostat under 'steady state' conditions where the composition of the environment, rates of nutrient consumption and of bacterial synthesis are invariant with time. Innumerable 'steady states' are possible, depending on such factors as the composition of the medium, incubation temperature and dilution rate. A systematic study of the influence of each of these parameters on the culture viability, the weight of bacterial matter (biomass), ratio of biomass produced to growth-limiting nutrient consumed (yield) and number of organisms per ml. culture (bacterial concentration) can provide much information on the dependence of bacterial structure and function on environment.

Although assessments of bacterial concentration, viability and biomass can be readily made, and accurately reproduced, they are frequently not wholly meaningful in terms of the growing culture. For example, a measurement of biomass usually involves weighing organisms which have been separated from the culture constituents, washed with water and dried at 100°; but water and some of the substances removed from the organisms by the washing procedure are essential components of the cell and may vary considerably with the growth condition. Similarly, measurements of culture bacterial concentration and viability generally assume that every observable bacterial particle is a separate organism whereas this is seldom true, and the distribution of numbers of organisms per particle may also vary with the growth condition.

In some circumstances the yield value may provide more meaningful information than values for either bacterial concentration or biomass. Thus, when the growth rate of *Aerobacter aerogenes* was limited by the availability of NH_4^+, SO_4^{2-}, PO_4^{3-}, Mg^{2+} or K^+, the yield value varied considerably with dilution rate. Because each growth-limiting ion was almost completely taken up from the environment and retained within the organisms at all dilution rates below about $0.8D_c$, these yield variations indicated a progressive change in bacterial nitrogen, sulphur, phosphorus, magnesium, and potassium content, respectively, with growth rate. The changes in nitrogen content of NH_4^+-limited *A. aerogenes* and of sulphur in SO_4^{2-}-limited organisms was due to storage of glycogen which varied in amount with growth rate, and thereby diluted the nitrogen and sulphur containing components of the cell to a greater or lesser extent. Changes in yield value of PO_4^{3-}-, Mg^{2+}-, and K^+-limited cultures with dilution rate were not due to storage of glycogen, or any other 'storage' compound, and therefore had some further significance.

Since the proportionate changes in yield value with dilution rate for PO_4^{3-}, Mg^{2+} and K^+-limited *A. aerogenes* cultures were identical, the variations in cellular phosphorus, magnesium and potassium content with growth rate must have also been identical. Because the magnesium content of Mg^{2+}-limited *A. aerogenes* organisms was stoichiometrically related to their RNA content (Tempest, D. W., Hunter, J. R. & Sykes, J. (1965), *J. gen. Microbiol.* **39**, 355) it seemed possible that variations in cellular phosphorus and potassium were also primarily linked with changes in bacterial RNA content; it has been recently established that the phosphorus, magnesium, potassium and RNA contents of K^+-limited *A. aerogenes* (and also of PO_4^{3-}-limited and Mg^{2+}-limited organisms) are stoichiometrically related to one another in the growing organisms.

Further evidence, from studies of yield variation with temperature, indicates that the above relationships may be mainly linked with the synthesis of functional ribosomes, the intracellular concentration of these also being a property of the growth rate (Ecker, R. E. & Schaechter, M. (1963), *Biochim. biophys. Acta* **76**, 275). Thus, varying the incubation temperature of *A. aerogenes* cultures growing at a fixed dilution rate caused the ribosome

content of the organisms to vary (Tempest, D. W. & Hunter, J. R. (1965), *J. gen. Microbiol.* **41**, 267), and the intracellular phosphorus, magnesium and potassium contents to change in parallel. Because phosphorus is a component of RNA, and magnesium and RNA are integral parts of the ribosome structure, the variations in bacterial RNA, phosphorus and magnesium with growth rate are readily understood. Our data suggests that potassium is a fourth component of this system and may act by maintaining the ribosomes in the precise configurational state, or degree of aggregation, necessary for them to function optimally.

Methods for Measuring Respiration and Dissolved Oxygen Tension in Growing Cultures. By D. HERBERT (*Microbiological Research Establishment, Porton, Wilts.*)

The two best-known methods for measuring microbial respiration, namely the manometric and the polarographic, are essentially methods for use with resting cell suspensions and are not well adapted for measuring the respiration of growing cultures, at any rate for periods of more than a few hours. This is because they are both 'closed system' methods in which the bacteria are confined in a vessel with a limited amount of oxygen; the technique of measurement, whether manometric or polarographic, depends on the fact that the amount of oxygen present is continually decreasing.

A method better suited to the study of growing cultures might be described as the 'Continuous Gas Analysis' method; this is an 'open system' in which a stream of air (or suitable gas mixture) is passed continuously through the vessel containing the growing culture and the change in chemical composition of the gas is continuously measured. Measurements can be continued for any length of time, and the method is particularly suitable for continuous culture experiments, though equally applicable to batch cultures.

Different applications of this method have varied in the gas chosen for measurement and the methods used to measure it. In principle, respiration is best determined by measurement of oxygen uptake, but it is legitimate to measure production of carbon dioxide when this is known to originate entirely from respiration and not from aerobic fermentation. Ideally one should measure both, which is not difficult (though expensive) if automatic gas-analysing instruments are available.

Both chemical and physical methods of gas analysis have been used. Undoubtedly the cheapest method is to take samples from the gas stream and analyse for oxygen and carbon dioxide by classical chemical methods, using, for example, the Orsat or Haldane apparatus, or such modern micro-versions of these as the Scholander apparatus. This is quite suitable for continuous cultures running in a steady state when the composition of the effluent gas stream is constant, but when the respiration is continually changing the number of samples requiring analysis becomes excessive, and some automatic system becomes essential. Fully automated apparatus for chemical gas analysis is available, but this is even more expensive than physical methods, which in any case are to be preferred as they give a truly continuous record.

Physical methods for analysis of carbon dioxide are based on measurement of infrared absorption or thermal conductivity; the former is more specific though more expensive (production of hydrogen by some bacteria can give rise to large errors in thermal conductivity methods). The most specific methods for oxygen measure its paramagnetism, since no other paramagnetic gas is likely to be present; available apparatus is based either on the 'magnetic wind' or the 'Pauling dumb-bell' principle.

Examples will be given of the use of these techniques, and the interpretation of measurements obtained with them. In this respect, measurement of the dissolved oxygen tension in microbial cultures is important, since cell respiration is markedly affected by the oxygen tension.

In principle, the dissolved oxygen in bacterial cultures can be determined by chemical analysis; in practice, the method is beset with sampling difficulties and most people prefer to use some type of oxygen electrode. Many types of electrode have been tried, most of which have disadvantages so far as microbiologists are concerned. Noble metal electrodes

(e.g. gold, platinum) rapidly become poisoned in contact with microbial cultures. The dropping-mercury electrode avoids this by constant renewal of its cathode, but is hard to sterilize and its susceptibility to convection currents precludes its use in stirred and aerated cultures.

The most common type of electrode in use at the present time is some type of membrane-protected electrode, i.e. an electrode enclosed within a membrane through which oxygen can diffuse but water and other solutes cannot. These are slow-responding, but are not susceptible to poisoning and are free of the pH limitations of 'naked' metal electrodes. Two types are in current use: (1) those based on the Clark electrode and containing an oxygen-reducing cathode in contact with the membrane (e.g. platinum, gold or silver) and an inner reversible electrode (e.g. Ag/AgCl); these require an external source of potential (about 0·7 volts) which must be highly stabilized; (2) the galvanic cell type of electrode with a consumable anode (e.g. of lead) which generates its own internal E.M.F. and requires no external source of potential; of these, the design of Mackereth appears the best currently available.

The availability of such electrodes, despite their present imperfections, has made possible the study of the effect of oxygen tension on cell respiration; moreover, by making such electrodes the sensing device of a controller which regulates the dissolved oxygen tension in a culture (such a device may be termed an 'oxystat'), bacteria may be grown in environments of any desired oxygen tension. This opens up a wide field for quantitative studies on the effect of oxygen on microbial growth and physiology.

The Analysis of Growth Curves. By E. O. POWELL (*Microbiological Research Establishment, Porton Down, Salisbury, Wilts.*)

The mass growth rate of a bacterial population depends in a complex way on the character of the environment; the simplest expression of the dependence is to be found in the chemostat, which permits the determination of growth rate (μ) as a function of the concentration of a single ('limiting') nutrilite (s), other features of the environment being constant. Under steady-state conditions the growth of the organisms is balanced and their physiological state is unchanging.

The various formulae which have been proposed to represent μ as a function of s should strictly be held to apply only to such a state, since under transient conditions μ depends on the past history of the population and is not a unique function of s.

The growth rate itself is best thought of as a composite function, the product of factors q, the rate of consumption of the nutrilite in question, and Y, the yield. In a closed system,

$$q = -\frac{1}{x}\frac{ds}{dt}; \quad Y = -\frac{dx}{ds};$$

$$\frac{1}{x}\frac{dx}{dt} = \mu = qY,$$

where x is the mass concentration of organisms. Both q and Y may vary with s. The metabolic coefficient q appears to be a simpler function of s than is μ. Monod's (1950, *Annls Inst. Pasteur, Paris* **79**, 390) formulation of the dependence of μ on s is often practically satisfactory, but a number of gross deviations from it are known and these can be satisfactorily explained as resulting from variations in Y, contrary to one of Monod's primary assumptions. Four types of variation can now be recognized. Of these only one is at present susceptible of simple algebraic expression, viz. the variation due to 'endogenous metabolism'—a standing requirement for a supply of energy or replacement material (Herbert, D. (1958), in *Recent Progress in Microbiology*, ed. Tunevall, Stockholm: Almqvist and Wiksell).

The metabolic coefficient q can itself be analysed further. It is the product of two factors, say S and q_a. The factor S is directly dependent on s and has the approximate form

$$\frac{s}{K+s}.$$

The factor q_a, called the *potential metabolic activity*, depends only indirectly on s; it is analogous to the concentration of enzyme in the Michaelis–Menten equation, and can be measured directly (Tempest, D. W. & Herbert, D. (1965), *J. gen. Microbiol.* 41, 143). Thus we have so far

$$\mu = Y.q_a.S;$$

each factor on the right is a function of s, and in favourable cases the influence of each on μ can be distinguished. It is to be noted that q_a is defined (like Y) with respect to a particular substrate in a given environment, and only under conditions of balanced growth.

Finally we may consider the changes in μ which occur when s is not held constant. During and after a change in s, the value of μ is not determined solely by the instantaneous value of s, but by all the values it has taken in the past—the physiological state of the organisms, represented by q_a, responds only slowly to change; the effect on S is instantaneous. Thus q_a must be replaced by a functional of s (Volterra, V. (1931), *Theory of Functionals*, London: Blackie and Son, Ltd.), say

$$Q \begin{bmatrix} \xi = \infty \\ s(t-\xi) \\ \xi = 0 \end{bmatrix},$$

the *metabolic activity functional*. At present there exist only some qualitative indications as to the nature of Q.

This analysis can be exemplified by applications to the work of Tempest & Herbert (loc. cit.), Mateles, Ryu & Yasuda (1965, *Nature, Lond.* 208, 263), Beran, Málek, Streiblová & Lieblová (1966, in *Microbial Physiology and Continuous Culture*, London: H.M.S.O. in Press) and Powell (*ibid.*).

The Mode of Growth of Colonies of Bacteria and Fungi on Surfaces. By S. J. Pirt
(*Department of Microbiology, Queen Elizabeth College, London, W.* 8)

The growth of protists in colonies on a solid medium such as nutrient agar has not been analysed quantitatively. Consequently little or no significance has been attached to colony growth rates. Quantitative studies on the growth of surface colonies of filamentous fungi have established empirically the law that, if r = radius of colony at time, t and r_0 = radius at zero time, then

$$r = k_L t + r_0. \tag{1}$$

The constant k_L is known as the 'linear growth rate'. The theoretical basis of the law is obscure (Plomley, N. J. B. (1959), *Aust. J. Biol. Sci.* 12, 53). Since the fungal hypha grows only at the tip, that is, the growth is apical, k_L may be equated with the rate of extension of the leading hypha. However, there remains the question—what determines the *rate* of apical growth? Despite the lack of a sound theoretical basis, the value of k_L has been used as a parameter of factors affecting mould growth. In contrast, it seems there have been no quantitative studies on bacterial colony development except on lag before growth (Dean, A. C. R. & Hinshelwood, C. (1956), *Proc. roy. Soc.* B 146, 109). Outlined below, is a model aimed at accounting for the growth of a colony in terms of the basic growth parameters. It is immaterial to the model whether the organisms grow apically as filaments or as single dividing cells.

The case when growth is limited by the supply of energy source in an agar medium is considered. It is supposed that, initially, the organisms have excess of nutrients and consequently will increase exponentially at the maximum rate. The colony will expand

equally in all directions forming a hemisphere (growth down in to the agar is excluded) as shown in Fig. 1a. A gradient of nutrient concentration will develop under the colony. Eventually growth in the centre of the colony will be limited by the rate of nutrient diffusion from the agar. When this is so, the colony is represented by Fig. 1b. At the periphery of the colony, in the zone ABC, nutrients are present in excess and growth is expenontial. The section ABC will be a quarter circle. Owing to the Michaelis–Menten relation between growth rate and nutrient concentration, and the existence of maintenance energy (Pirt, S. J. (1965), *Proc. roy. Soc.* B **163**, 224) it is concluded that the growth rate at B falls rapidly from the maximum to zero. Consequently the colony height from B inwards will have the constant value, H.
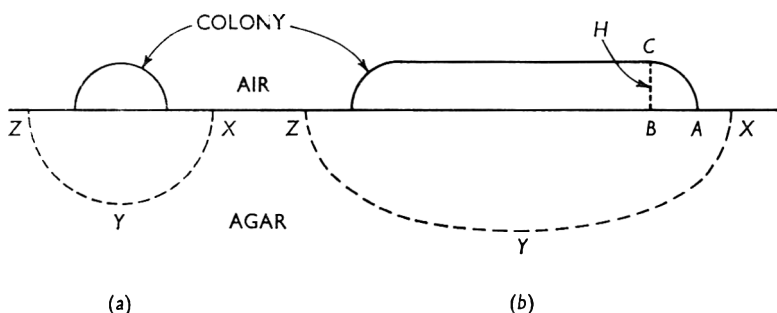
Fig. 1. Models of colony growth on agar surface. Vertical sections across diameters. (*a*) Exponentially growing colony. (*b*) Colony with linear growth rate. ABC represents the growing zone; XYZ is the contour of initial concentration of growth-limiting nutrient (not to scale).

Let v = volume of colony; v_g = volume of growing zone at the periphery; α = maximum specific growth rate $(1/m \cdot dm/dt$, where m = mass of organism at time, t). The growth rate of the colony is given by

$$\frac{dv}{dt} = \alpha v_g. \tag{2}$$

When H is small compared with r it is derived that

$$r = \tfrac{1}{4}\pi H\alpha t + r_0. \tag{3}$$

Thus, according to this model, the colony will have a constant linear growth rate given by

$$k_L = \tfrac{1}{4}\pi H\alpha. \tag{4}$$

In order to use relation (4) to compare specific growth rates, the dependence of H on the specific growth rate must be known. In principle, this can be determined from consideration of the change in nutrient concentration in the agar and the nutrient uptake by the colony. An approximate simplified treatment of this problem indicates that $H \propto 1/\sqrt{\alpha}$. Hence, $k_L \propto \sqrt{\alpha}$.

It has been verified that bacterial colonies, under the appropriate conditions, have a linear growth rate $\propto \sqrt{\alpha}$. The effect of concentration of growth-limiting nutrient will be considered. Also the extension of the theory to oxygen limitation and growth inhibitor effects will be discussed.

Measurement of Drug Action on Single Bacterial Cells. By L. B. Quesnel (*Department of Bacteriology, University of Manchester*)

All the methods to be considered here involve some form of microscopic observation, and can be divided broadly into two categories: (*a*) those in which it is not an essential requirement that the cells are alive at the time of observation, and (*b*) those in which the cells are maintained in a dynamic state under continuous observation. Class (*a*) techniques require no exceptional forms of instrumentation, while 'microcultural studies, particularly, present some formidable although generally unappreciated technical difficulties' (Hoffman, H. (1964), *Ann. Rev. Microbiol.* **18**, 111) which probably accounts for the almost total absence of their application to the study of drug action.

Relatively few properties of individual living cells can be quantitatively measured: lengths and widths can be measured fairly accurately and estimates made of volume, while changes in morphology, e.g. under the influence of penicillin, have been filmed many times. The number of nuclei per cell can be recorded by phase contrast microscopy (Knöll, H. & Zapf, K. (1951), *Zentbl. Bakt. ParasitKde. I.* Orig. **157**, 389) as well as generation times, genealogies and rates of clonal development. The definition of cell viability is precarious and demands a specific and restricted statement for any particular experimental situation, but with this understanding the bactericidal effect of drugs can be usefully estimated by direct observation in many cases.

In class (*a*) applications the cells may be allowed to react with the drug under the required conditions and samples removed at intervals for stained smears or wet preparations. From solid media impression preparations may be made or the cultures examined *in situ* under the microscope. Koopermans ((1960), *Antibiotics Chemother.* **10**, 612) and Mahoney & Chadwick ((1965), *Can. J. Microbiol.* **11**, 829) described rapid methods for the evaluation of antibiotic sensitivity by microscopic examination of drug–agar slide or plate cultures for presence or absence of microcolony development which give good correlation with paper disc determinations. Quesnel & Thornley (in Press) have applied a method similar to the microculture technique of Postgate, Crumpton & Hunter ((1961), *J. gen. Microbiol.* **24**, 15) to measure the synergistic effect of nalidixic acid and chloramphenicol at low antibiotic concentrations.

Class (*b*) techniques for prolonged observation have been reviewed by Hoffman ((1964), loc. cit.) and include hanging drop, hanging block, sealed agar block (Ørskov, J. (1922), *J. Bact.* **7**, 537), covered smear, slide chamber and Petri plate methods with dry objectives or under oil immersion by first covering the developing microcolonies with a coverslip. The covered smear technique (Fleming *et al.* (1950), *J. gen. Microbiol.* **4**, 257) has been used to study the morphology and motility of organisms under the influence of penicillin, while a similar method was used by Pulvertaft ((1952), *J. Path. Bact.* **64**, 75) to study the effect of penicillin, streptomycin, aureomycin, chloramphenicol and terramycin on a number of organisms. A modification was used by Adler & Hardigree ((1965), *J. Bact.* **90**, 223) to enable them to study the effect of Mitomycin C on the nuclei of filament-forming cells of *Escherichia coli*. Zapf ((1953) *Zentbl. Bakt. ParasitKde. I.* Orig. **159**, 436) studied the effect of ultrasonic treatment and penicillin on *E. coli*.

All these methods suffer from drawbacks due to a changing environment or involve unwanted 'shock' effects difficult to control. To overcome these Powell has devised an ingenious continuous flow chamber (Powell, E. O. (1956), *Jl R. microsc. Soc.* **75**, 235) which enables prolonged phase-contrast observations with the advantage that the medium can be changed at will enabling the study of short-time contact effects of drugs. The chamber has been used mainly for the study of 'normal' growth (Powell, E. O. (1958). *J. gen. Microbiol.* **18**, 382; Quesnel, L. B. (1963), *J. appl. Bact.* **26**, 127) rather than of drug inhibition, e.g. by streptomycin (Quesnel, L. B. (1962). *J. appl. Bact.* **25**. iii).

Two main problems arise in regard to continuous-observation studies: environmental control, especially temperature, and accurate recording. Solutions to the former are the use of temperature-controlled rooms for both equipment and operator or incubators which must reach a very high standard of design (Hoffman, H. & Frank, M. E. (1963), *J. Bact.* **85**,

1221). The best recording procedure is the use of reliable 35 mm. time-lapse photo-micrographic equipment—but even this does not solve the serious problem of steady focus and good definition.

The Measurement of Drug Action on Populations of Bacterial Cells. By K. E. Cooper and A. H. Linton (*Department of Bacteriology, Bristol University*)

Absorption of an antibiotic or a nutrient on cell receptors is the usual mechanism by which the substance is made available to the cell for the subsequent series of actions which modify cell growth. The measurement of inhibitory levels resulting from absorption of an antibiotic on to the test organisms varies according to the method of assay employed. Methods involving the incorporation of an antibiotic in the medium, and the use of *small* inocula, determine an end-point—the minimum inhibitory concentration—in which inhibition of growth is brought about by absorbed antibiotic in equilibrium with a concentration in the supernatant and this concentration is altered very little during the test. The initial inoculum (N_0) and the initial concentration (C) are in equilibrium with the amount absorbed (a/per cell).

Thus $N_0 + C \rightleftharpoons N_0 a$.

The use of growth curves to indicate the inhibitory effect of an antibiotic in batch culture with *small* inocula enables more quantitative estimates of bactericidal and bacteriostatic effects to be determined. When *large* inocula are used much greater amounts of the antibiotic are removed by absorption on to the organisms. This results in a correspondingly lower concentration in the supernatant.

In diffusion methods the critical concentration (m') (Cooper, K. E. (1963), *Analytical Microbiology*, ed. F. Kavanagh, p. 12, New York: Academic Press Inc.) leading to the formation of the edge of the inhibition zone is reached at a time (T_0) when the population has increased to a much larger size than is present in incorporation methods. This critical population (N') absorbs antibiotic from the diffusing critical concentration and reduces it to levels below the minimum inhibitory concentration ($m' = C + N'a$) and growth continues to occur. The difference between critical and minimum inhibitory concentration is thus a measure of the amount absorbed on the critical population; the amount absorbed per cell should be given by $a = (m' - C)/N'$.

The effects of pH and Mg^{2+} on the growth of staphylococci, and on the critical concentration of streptomycin have been studied. The competition of hydrogen ion and magnesium for the cell receptors able to absorb streptomycin has been investigated, and the effects of inoculum size and the resistance of a number of staphylococcal strains compared under conditions in which critical and minimum inhibitory concentrations of streptomycin can be determined.

Plotting the effect of added excess magnesium salts (log molar concentration) on the critical concentration (log m') gave straight line relationships (at each pH) above log $Mg^{2+} = 2 \cdot 7$ (i.e. $0 \cdot 05$ molar conc.) suggesting direct competition for receptors by streptomycin and Mg^{2+}. As the pH is lowered (range $8 \cdot 0$ to $5 \cdot 5$) the critical concentrations increase and the plots for each pH level are parallel. Analogous plotting of the effect of pH on critical concentration at differing Mg^{2+} levels similarly suggests competition between hydrogen ion and streptomycin, since the plots of log m' are increased in parallel for each level of added magnesium (for pH values below $7 \cdot 8$). There appears to be an optimum availability of receptors for streptomycin at approximately pH $7 \cdot 8$ and concentration of magnesium below $0 \cdot 05$ molar. We have found that the effects of magnesium on the lag period in cell division, under conditions allowing full metabolism, depend on inoculum size. Very long lag periods with little increase in eventual generation times can be produced by excess magnesium in batch culture, especially with small inocula. The lag period is little affected by inoculum size under optimal conditions. Hinshelwood (Hinshelwood, C. N. (1946), *The Chemical Kinetics of the Bacterial Cell*, p. 41. Oxford: Clarendon Press) showed that it was increased under conditions of magnesium deficiency, but our basal medium has adequate magnesium.

Measurements of population changes resulting from drug action therefore necessitate

the precise definition of conditions under which they are made. It is particularly important to define the condition of the receptors on the organism, and the factors in the environment that may influence these conditions.

Temperature and Concentration Coefficients in Disinfection. By G. G. MEYNELL (*Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, London, S.W.* 1)

Microbial killing has often been considered as a pseudo-first order reaction, largely because of the frequent occurrence of exponential survival curves. Regardless of whether this view is justifiable on biological grounds, its predictions are convenient for expressing the effect on killing rates of change in temperature or in disinfectant concentration. These will be illustrated by examples from the literature and the significance of the parameters discussed.

Index of Authors

Small roman numerals refer to pages in the Proceedings of the Society for General Microbiology

Index of Subjects

Small roman numerals refer to pages in the Proceedings of the Society for General Microbiology