SIR PAUL FILDES

(*Frontispiece*)

J. gen. Microbiol. (1962), **27**, 355
Printed in Great Britain

The 80th Birthday of Sir Paul Fildes

Sir Paul Fildes was born on 10 February 1882 and is still an active microbiologist. The Committee of the Society for General Microbiology dedicates this issue of its Journal in honour of Sir Paul on the occasion of his 80th birthday.

Sir Paul has devoted a life-time to the study of pathogenic bacteria. As a bacteriologist in the London Hospital Medical College from 1909 and concerned with naval medicine during the war of 1914–1918, Sir Paul studied the chemistry of bacterial growth. He saw that to control the growth of microbes it is necessary to know how they convert materials of their environment into more of themselves and into products of their metabolism. This meant combining the disciplines of classical bacteriology and biochemistry. First at the London Hospital and later at the Middlesex Hospital, under the auspices of the Medical Research Council, Sir Paul organized groups of workers to carry out this design. As a result of their work certain principles emerged which were seen to be of general applicability for other biological forms. This work initiated and organized by Sir Paul contributed significantly to the recognition that there is an underlying unity in all biochemistry at the cellular level. It also showed that the study of bacterial physiology can contribute much to the discovery and analysis of metabolic pathways involved in biological growth in general and to methods by which such growth may be curtailed by chemotherapeutic and antiseptic agents; work on the modes of action of sulphonamides and of mercury salts led to the proposal of his rational approach to research in chemotherapy. The methods of finding pathways through the jungle of microbial physiology, as prospected by the groups organized by Sir Paul, have now become common in wide fields of biology.

Throughout his research life Sir Paul has remained a bench-worker, a meticulous technician. He took a large part in organizing and carrying through the production of the monumental *System of Bacteriology* published (1930–1931) by the Medical Research Council. He is the founder (1920) and director of the *British Journal of Experimental Pathology* which served as the main vehicle of publication for the work of the Fildes school. Sir Paul has played a cardinal role in shaping microbiology as we know it today; he is one of the fathers of modern microbiology.

B.C.J.G.K

CORRIGENDUM

In LEINWEBER, F.-J. & LIVERMAN, J. L. (1961)

J. gen. Microbiol. **26**, 529–533

On p. 532, line 11:

for: '...homocysteine is shown to be incomplete if the incubation period...'

read: '...homocysteine becomes incomplete if the incubation period...'

J. gen. Microbiol. (1962), 27, 357–372
With 1 *plate*
Printed in Great Britain

The Growth of Microbiology

The Fifth Marjory Stephenson Memorial Lecture

By B. C. J. G. KNIGHT
Department of Microbiology, University of Reading

...I melt a gourd fruit into mash,
Add honeycomb and pods, I have perceived,
Which bite like finches when they bill and kiss,—
Then, when froth rises bladdery, drink up all,
Quick, quick, till maggots scamper through my brain....

Robert Browning, *Caliban upon Setebos*

I am deeply conscious of the honour of being asked to give this fifth Marjory Stephenson Memorial Lecture; and I am even more conscious of the difficulties of the task. I think that it might be appropriate, sixteen years after the founding of our Society, to look at the origins and growth of the science of general microbiology from which our Society takes its name. History gives perspective and an all-round view which is especially useful in the case of microbiology, with its manifold branches and applications. If not held together by a general conception, microbiology might lose much of its strength and effectiveness.

In attempting to sketch the history of microbiology I have selected what I think are some key phases in its development. I am well aware that in so doing I shall make generalizations which may be questioned. Microbiology grew from many sources, and it would need large tomes to describe in detail its developmental history. All I hope to do is to indicate some of the paths by which we have arrived at the present state of our science.

When one tries to trace the history of a branch of science, the more one reads the more one finds that advance does not come by a logical sequence of steps from man to man, as it were. Mistakes of interpretation, and hypotheses which are subsequently disproved, have their value if they stimulate experiment. In order to abridge, it is inevitable that some individuals will be named, as typical or as having been responsible for key work. As we advance in time, the interactions of the work of different people become more and more complex; and in the present century we find collective work by groups, and more complex systems of communication.

I give this preamble to protect myself from being thought unaware of the great intricacy of the history of microbiology. But also to apologize to, and to pay tribute to, all those in the past and in the present who are not mentioned by name, but whose work has contributed to shape microbiology. This lecture can be only a bare sketch, but I hope an impartial one, although others might give different emphases to different parts of the story. Much of what I say will be familiar; for this I ask your indulgence.

Vol. 27, *No.* 2 *was issued* 13 *February* 1962

Origins of microbiology

The origins of microbiology may be found in those numerous natural phenomena which are manifestations of microbial activity and which men used, or noticed, or suffered, without knowing that they were the effects of microbial growth. One can group these effects under several heads: (1) arts and crafts based on microbial activities; (2) soil fertility; (3) spoilage of stored products; (4) animal and plant diseases. These are ways in which micro-organisms have drawn attention to themselves.

(1) *Arts and crafts.* The alcoholic fermentation of fruit and plant juices which occurred apparently spontaneously, because of the presence of unsuspected yeasts, was one of the earliest instances of microbial activity of which men made use; and similarly the souring of drawn milks by the action of lactic acid-forming bacteria which found milk an excellent elective culture medium.

Beer-making is more complicated since it involves two stages: the preparation of malt by allowing cereal grain to germinate, thus changing starch to sugar; and the fermentation of the sugar by ubiquitous yeasts. There are Egyptian records of beer-making by this process as early as 2000 B.C. In a Chinese agricultural encyclopaedia of the sixth century A.D. (see Davis, 1945) there are examples, in connexion with domestic economy, of the use of so-called ferment-cakes which must have contained both moulds and yeasts, the combined action of which gave a wine in one process from steamed rice. In beer-making, in the use of ferment-cakes and in the use of leaven from a previous batch of dough in bread-making, the principle involved was that a separated portion of material from one batch, and which could be stored, was used to inoculate a new batch. This is far more sophisticated than those processes which rely on a naturally accompanying microflora, like the yeasts on grapes in wine making.

(2) *Soil fertility.* Micro-organisms of course play an essential part in decomposing dead plant and animal materials so that the elements contained therein are returned as simple compounds to the soil, and in the fixation of atmospheric nitrogen. Early agricultural practices such as allowing soil to lay fallow and the planting of leguminous crops made use of these microbial activities.

(3) *Avoidance of spoilage.* The preparation from milk of fermented and soured products, and the making of cheese, are examples of microbial action which gave food products more durable than the raw starting material. Other methods of food preservation in early use involved the provision of environmental conditions unfavourable to microbial growth, such as desiccation, high salt content, low temperatures, unfavourable pH values.

(4) *Animal and plant diseases.* Epidemic diseases of man were unavoidably noticed and their characteristics recorded. The similarity of symptoms in given epidemics bred theories about the operations of generally-acting evil forces. One has only to look at the many words in the English language which have been used to denote these sorts of effects: plague, pest, murrain, blast, smut, rust, mildew, mould, rot, putrefaction, blight and so on, to realize how forcibly these manifestations were noted and distinguished.

In speculations about the causes of epidemic animal diseases, as in the great plagues, the question of infection or contagion, as it was originally called, was much

considered. As early as 1546 Fracastoro published a book, *De contagione...*, in which he discussed contagion. He clearly described and discussed several diseases, including foot-and-mouth disease in cattle, measles, smallpox and typhus. He compared the spread of these diseases with his observations of the spread of a rot or mouldiness through stored fruit or from fruit to fruit on the plant. But he did not consider the contagious agents as particulate, like the dust from moulds. Fracastoro's book to all intents and purposes had no immediate effect.

There are some lines in *Hamlet* which show that Shakespeare was a good observer who might have made a good microbiologist. These lines occur in the scene when Hamlet reproves his mother for her marriage with his murdered father's brother. He compares the two brothers, and says:

> This was your husband. Look you now, what follows:
> Here is your husband: like a mildew'd ear
> Blasting his wholesome brother.
>
> (Shakespeare, *Hamlet*, Act 3, Scene 4.)

Here is a typically Shakespearean play upon words, the father's ear filled with poison, and the infected ear of wheat blasting its neighbour. What clearer description could one want about the transmission of infection by contact?

The date of *Hamlet* is about 1600, some 50 years after Fracastoro's book which probably Shakespeare never read, and more than 50 years before William Boghurst wrote his manuscript book *Loimographia* (1666) in which he described his observations during the Great Plague of London in 1665, recording very acutely what he saw. However, as Bulloch (1938; p. 13) pointed out, Boghurst expressed opinions about the causes of the epidemic which were 'not very far removed from those of the ancients, but which were very different from that urged by Fracastorius 126 years earlier'. This is a very early and perhaps understandable failure of communication in the history of microbiology.

The next great step was, of course, the work of Leeuwenhoek (1632–1723) in showing the existence (1676, 1683: see Dobell, 1932) of such small organisms that the microscope was needed to see them. But it was a long time before micro-organisms were connected with any of the phenomena of microbial growth which I have mentioned. From the time of Leeuwenhoek onwards there was a great development in the construction of microscopes and in the observations made by their aid. The use of the microscope as the main tool for examining these small organisms made it inevitable that their varied shapes should first receive most study. Thus it was that the larger motile forms, the protozoa, should become the prey of zoologists. Other large forms, of filamentous appearance and much less agile habit, were annexed by botanists, to whom fell also the small green algal forms.

Suggestions that micro-organisms such as Leeuwenhoek had described might be agents in any of the phenomena which I have listed were made only more than a century later. Mathieu Tillet (? 1714–91), by a remarkable series of plot experiments, showed (Tillet, 1755) that the disease of wheat called bunt or stinking-smut was transmitted by contact of seed corn with the black dust from the bunt-balls which replaced the healthy grain in diseased ears. He also showed that treatment of seed corn with various alkaline washes decreased the incidence of the disease.

He examined the black dust microscopically and saw minute spherical bodies of uniform size which he thought were possibly analogous with the dust from puff-balls; but he did not recognize them as fungal spores. In a memoir published in 1807 Bénédict Prévost showed that the black dust of the bunt-balls could grow. With the microscope he watched the germination of the spores in water suspension and said that the minute spherical bodies of the bunt dust were the seeds or spores of a microscopic plant, and that it was this living organism which caused the disease of the grain. He also described the lethal effect of copper salts on these spores.

The potato murrain or potato blight which devastated potato crops in Ireland in 1845 and after, and in Europe, and a disease of grape vines first noticed near Margate in the same year which devastated vines in France in the following years, were said by M. J. Berkeley (1846; see Large, 1946, chaps. 2, 3) to be fungal diseases. Although many observers concurred in finding a given fungus associated with the same kind of disease in a given kind of plant, still the evidence remained only circumstantial that a fungus was the cause of a disease. But it became less and less easy to maintain that the fungal growth only followed a weakening of the plant, brought about in some other way which was the real cause of the disease.

The observation of the existence of a micro-organism capable of entering the animal body and there multiplying to cause disease was recorded by Agostino Bassi (1773–1856) in his now celebrated book *Del mal del segno*...(published in 1835) about one of the diseases of silk worms then causing great losses to the silk industry in northern Italy. From many experiments Bassi came to the conclusion that the disease was really contagious. He transmitted the disease from sick larvae to healthy ones by a technique which involved removing a portion of epidermis from a sick silk-worm, flaming the larva, touching the underlying tissues with a previously flamed pin, and then scratching a healthy silk-worm with it. Bassi recognized the causative organism, which formed the characteristic white efflorescence on the larvae after death, as a fungus. It is understandable that it was fungi, giving discernible growth on the host, which should first have been recognized as probable causes of certain animal and plant diseases.

P. F. Bretonneau (1778–1862) was the founder of the doctrine of the specificity of animal disease. He deduced from many clinical and pathological observations that it was the nature of the morbid cause rather than its intensity which explained the different pictures presented by different diseases. Bretonneau (1826) thought that diphtheria and typhoid fever developed under the influence of specific contagious principles or reproductive agents. He did not, however, connect these reproductive agents with any of the microscopic forms which were well known and much discussed by microscopists in Bretonneau's time.

The demonstrations of Prévost and Bassi of fungal diseases in plants and silk worms, respectively, appear to have had little immediate effect in making easier the acceptance of a germ theory of animal, particularly human, disease, although their work, together with that of Cagniard-Latour, Schwann and Kützing on the connexion of yeast as a living organism with fermentation, in the 1830's, and the theoretical conclusions of Henle (1840), stimulated the microscopical examination of pathological materials. Various human dermatophytes were described during the period 1840–50 (e.g. Schönlein, 1839; Gruby, 1842, 1843, 1844; see Ainsworth, 1951; Bulloch, 1938). But it needed the work of Pasteur (beginning 1857; see bibliography)

on the role of bacteria in the causation of specific biochemical effects, and that of Robert Koch (1878) on anthrax, before the role of bacteria in animal disease was finally accepted.

Biochemistry

It is convenient at this point to turn to that other main stream of study which eventually fused with microbial studies, namely the chemistry of biological *compounds*, which led to the chemistry of biological *processes*, to biochemistry. From the earliest records there is evidence of the chemical study of biological products, no distinction being made between inorganic and organic materials. In the seventeenth century chemistry developed in two directions typified by Agricola the father of metallurgy, and by Paracelsus the founder of medical chemistry. Paracelsus used mercury and inorganic mercurial compounds in the treatment of syphilis—the line which Ehrlich developed successfully so many years later. Some of the followers of Paracelsus tried to extract from biological materials their active medicinal principles; many essential oils were separated, and numerous simple organic compounds obtained by dry distillation.

By the end of the eighteenth century organic compounds began to be investigated from a new point of view, i.e. as substances in themselves rather than as substances for use in pharmacy or in various arts and crafts. Lavoisier (1789) investigated vinous fermentation and showed that the conversion of sugar into ethanol and CO_2 could be expressed quantitatively. This was something new and marks an important step in the chemical investigation of biological processes.

From the beginning of the nineteenth century organic chemistry emerged as a well defined science, concerned with the development of chemical theory, the determination of structures, and the synthesis of carbon compounds, many derived from biological sources. After its coal-tar debauches organic chemistry returned once more to Mother Nature, to study the most complex components of the chemical anatomy of the cell.

In the study of fermentation and putrefaction the chemistry of biological compounds began to change into the chemistry of the *processes* whereby they were formed, into biochemistry. The study of the chemical effects of micro-organisms began in pronounced form with the controversy about the nature of yeast and its action in fermentation. In the 1830's Cagniard-Latour (1836), Schwann (1837) and Kützing (1837) independently concluded that yeast was a living organism, and performed experiments which implicated the yeast granules as the cause of vinous fermentation. The theory of the biological causation of fermentation developed by these and subsequent workers met strong opposition from chemists who were loath to admit that fermentations were the result of biological activity. It must be remembered that this work was taking place in a context where chemistry was beginning successfully to determine the structures of biological compounds and to make them by purely chemical reactions. 'Vital forces' were retreating before chemistry.

By the middle of the nineteenth century the conditions were ripe for the fusion of the two streams I have indicated: on the one hand, the study of phenomena potentially caused by micro-organisms; and on the other, the chemistry of biological products and the processes whereby they are formed. Undoubtedly if Pasteur had

not arrived at that time this fusion would have taken place. Too many men were then working as professional scientists for it to have been long delayed. But Pasteur did arrive, and there is no clearer example of the right man at the right time. The fusion took place and there came that explosive reaction from which microbiology, the science of microbes*, was born.

Aseptic technique

Before continuing to describe the evolution of microbiology which began with the work of Pasteur it is convenient to intercalate a brief consideration of the question of spontaneous generation or heterogenesis, since the experimental study of this rather metaphysical question led to the establishment of sound aseptic techniques —a crucial point in the growth of microbiology. Whether new living forms might appear from non-living materials was a very ancient question. The publications on this topic by Redi (1668), Joblot (1718), Spallanzani (1765), Schwann (1837) and others contributed greatly to defining the experimental conditions necessary to prepare and maintain sterile nutrient infusions. This work culminated in that of Pasteur and John Tyndall which showed that microbial growth in heat-sterilized nutrient infusions occurred only when defects in technique permitted viable particles carried by the air to enter vessels containing the heat-sterilized media. One of John Tyndall's important contributions was the demonstration of the greater heat tolerance of some bacterial forms (spores) as compared with other (vegetative) forms. His method of sterilizing media by intermittent heating, with intervals to allow heat-tolerant spores to germinate to heat-sensitive forms, was worked out then; this procedure we now call Tyndallization. Some of the apparatus with which Tyndall's work was done here is exhibited in the gallery which surrounds this lecture theatre. His classic book *The Floating Matter of the Air* was published in 1881.

The practical outcome of this work on the question of spontaneous generation or heterogenesis was the realization of the ubiquity of viable minute air-borne forms and the establishment of aseptic techniques whereby experiments could be made under controlled conditions, so that chance contamination should not vitiate them. This provided an essential technical basis for the advance of microbiology.

Microbiol physiology

I turn now to the development of microbial physiology stemming from the work of Pasteur. Pasteur, trained as a chemist, made his first contact with micro-organisms through his efforts to resolve the racemic mixture of D- and L-tartaric acids, when he observed that a mould was capable of decomposing one component only of the racemic mixture, leaving the D-form undecomposed. Thus the chemical problem posed by the enantiomorphs of tartaric acid, itself a product of wine-making, brought Pasteur in direct contact with a beautiful example of specific microbial action. His interest in optical activity lead him to study optically active

* The word 'microbe' was coined by the surgeon C. E. Sédillot (1878) to designate any organism so small as to be visible only under the microscope. Pasteur rarely used this word, preferring to use 'micro-organism'. He suggested later that the science of microbes might be called 'microbie' or 'microbiologie' which he regarded as having a wider meaning than 'bactériologie' (see Bulloch, 1938, pp. 187, 397; Dubos, 1950, p. 188).

amyl alcohol, another fermentation product, and this led him to study those fermentations which yielded lactic, acetic and butyric acids. As it happened, these fermentations were caused by bacteria. Through his work the causal connexion of the chemical action with the growth of specific bacteria was firmly established. Bacteria as agents of chemical change came to the fore.

Soon after, bacteria were shown to be agents of animal disease by the work of Robert Koch and of Pasteur and his associates in the 1870's, on anthrax. Then it was that Koch applied in practice the criteria to be used in order to prove that a given organism was the causative agent of a given disease, subsequently referred to as 'Koch's postulates'; the conditions had in fact been stated theoretically by Henle in 1840 (see Fildes & McIntosh, 1920).

During the next 20 years, after the proof that anthrax bacilli were the cause of that disease, many more bacteria pathogenic for animals were discovered, in particular by the school led by Koch, whose techniques for the isolation and identification of bacteria (e.g. plating separated viable units on the surface of culture media solidified at first by gelatine, later with agar-agar) were cardinal developments in bacteriology. There is no need to expand this here, except to suggest that the importance of this diagnostic aspect of bacteriology, in connexion with medicine, resulted in a departmentalization which, on balance, retarded the study of the organisms themselves.

I wish now to consider some of the general results of Pasteur's fermentation studies. The important conceptual step was that Pasteur looked upon the chemical activities of micro-organisms as directly related to their physiological processes—the processes whereby they lived and multiplied. That the chemical compounds which had been the initial point of interest were the result of bacterial action was one thing, but that these compounds were essential products of the metabolism—the biochemical processes of multiplication—of the organisms was a much more pregnant idea. It was this conception that Pasteur maintained against the opposition of chemists led by Liebig (see for example, Liebig, 1840, 1870), who held that the ferments involved were merely catalysts, perhaps produced by, but not causally connected with, the growth of cells. It was not until after Pasteur that the apparent contradiction between the two conceptions was resolved.

To summarize the effect of Pasteur's work in microbial physiology I cannot do better than quote what Marjory Stephenson wrote in her paper read at the Congress held in 1946 to commemorate the 50th anniversary of Pasteur's death; she wrote:

> Amongst the many remarkable aspects of this wonderful man is the attitude he adopted in the biological interpretation of the chemical effects produced by microbes. We are most of us familiar with the attitude of the chemist when he turns to microbiology for the solution of his problems; he then expects to use his microbes as a reagent but almost always neglects to study the problem from the point of view of the cell itself. Many chemists never attain the biological attitude, others only after long and painful experience. How different was the case of Pasteur who admittedly turned to fermentation to solve a problem in sterioisomerism but immediately adopted in his studies a most pronounced biological viewpoint. Fermentation was not regarded as a problem in isolation but as the chemical expression of a method of life—*la vie sans air*. Every other chemical activity of the organisms as it was disclosed was related to cell physiology and thus was the foundation of bacterial metabolism well and truly laid.
>
> Such was the prestige and vigour of Pasteur that he permeated the thought of his time

and directed it along his own lines. Nitrification, nitrogen fixation, sulphur and iron oxidation and all the fundamental discoveries of the Pasteur period were interpreted in terms of cell physiology and there can be no doubt that the surprisingly rapid advance made between 1858 and the end of the century was due to this outlook.

In spite of the fact that what may be called the Pasteur outlook stimulated so much brilliant discovery, it took little account of the rapidly accumulating knowledge of enzyme action to interpret the physiological processes involved. One can get an excellent conspectus of the state of knowledge about enzymes at the end of the nineteenth century from Reynolds Green's book *The Soluble Ferments and Fermentations*, published in 1899. The following quotation from the preface to this book indicates the quality of biochemical thinking about enzymes at that time:

> The more recent work takes the subject beyond the stage at which it was left by Pasteur, showing us that precisely similar operations are incidental to the life of the higher organisms. It thus becomes necessary to inquire into the relationship of protoplasm to metabolism, and to the association of ferments or enzymes with the living substance, and so to establish the intimate relationship of fermentation to the ordinary metabolic processes. It becomes possible to go further than this, and to consider by what chemical or physical processes the observed changes or decompositions are affected by protoplasm or by its secretions.

This foreshadowed a comparative biochemistry which only began really to develop much later.

When Reynolds Green was writing there were appearing in Paris, in the years 1898 to 1902 successively, the four large tomes, in all about 3000 pages, of the *Traité de Microbiologie* written by Émile Duclaux, Pasteur's chemical colleague and his successor as Director of the Institut Pasteur. Duclaux's *Traité de Microbiologie* shows uniquely what microbiology was at the beginning of the twentieth century. These four volumes collected and organized the very large amount of work in the field of chemical microbiology which had been accomplished up to then. I am amazed, in reading them, not only by the volume and quality of the work which Duclaux organized, but by the modern tone of his synthesis. In many respects the tables of contents read like the headings for a course of modern microbiology. It seems to me extraordinary that this great work should not have had a greater impact in effecting an earlier fusion between biochemistry and the more biological aspects of microbiology than in fact took place.

The demonstration by E. Büchner in 1897 that yeast cells could be broken to yield a juice which could perform alcoholic fermentaton was a key step in enzyme studies. This brought the study of fermentation into a new field of experiment: the study of cell-free biochemical processes. The school of Harden (1911 and after) in Britain and the largely German-led schools in Europe gave the great advances in our knowledge of the processes of glycolysis which took place in the second and third decades of the present century. But as Marjory Stephenson (1946) went on to comment in the paper from which I have already quoted:

> It is moreover noticeable that in periods, such as that immediately following Büchner's achievement, when the study of cell physiology tends to be disregarded, general microbiology advances only slowly, and within my own personal experience the same holds good; microbiology can only be successfully pursued as the study of a cell in relation to its environment, whether that environment be the outside world or the tissues of the host animal or host plant.

When Duclaux wrote his treatise at the beginning of the twentieth century he was conscious of the rate of development of microbiology, and of the future impossibility of any one man writing such a book. In the preface to vol. I (1898) he said:

> ...and when one thinks that microbiology is joined by the study of enzymes to one of the most unknown regions of chemistry, by the study of proteins to one of the most difficult, by the study of the microflora of earth, air and water to general hygiene, by the study of fermentation to the whole of physiology, by that of viruses [pathogenic microbes] and poisons to the whole of medicine, one cannot but conclude that the day is near when a microbiologist ought to be at one time so many things that this will no longer be possible; he will have to choose.

In fact, workers with micro-organisms did choose, and there was rapid and successful study of many fields of microbial activity. This led to a fragmentation of microbiological studies into separate fields of application, with consequent difficulties of inter-communication and a stress on the results of microbial action rather than on the physiology of the micro-organisms whereby they multiply in their environments. The study of microbial physiology I take to be a more basic and unified study than the study of the consequences—viewed anthropocentrically—of microbial growth, which reflect only some aspects of physiology. As Kluyver (1956) remarked:

> Since Pasteur's startling discoveries of the important role played by microbes in human affairs, microbiology as a science has always suffered from its eminent practical implications.

This fragmentation of microbiology into many fields of application helps to explain the lag in the integrated study of microbial physiology which took place after Pasteur's work. Marjory Stephenson deliberately chose bacteria as tools for biochemical studies, in order to use organisms 'more susceptible to laboratory control'. From 1920 onwards her school laid the basis of the modern study of bacterial metabolism. Her book *Bacterial Metabolism* (1930) was fundamental in co-ordinating and stimulating work in this field, with its stress that metabolic enzymes could not

> profitably be considered apart from the systems of which they form part i.e. from the action of other enzymes and also from that still mysterious mechanism by which the cell co-ordinates its separate systems in its own service. (Introduction to *Bacterial Metabolism*, 1930, p. 9.)

Microbial nutrition

The study of bacterial nutrition also gained momentum in the 1920's. From the time of Pasteur some fungi and yeasts had been grown in simple defined media, but it was attempts to grow certain animal pathogenic bacteria in laboratory culture which focused attention on their exacting nutritional requirements. Two examples may be mentioned.

Twort & Ingram (1913) found that Johne's bacillus, *Mycobacterium johnei*, which causes a chronic enteritis in cattle, could not be grown in complex bacteriological media unless these were supplemented with products of other mycobacteria which could grow in the unsupplemented media. In the 1920's work on the laboratory cultivation of *Haemophilus influenzae* showed that this organism would not grow in autoclaved complex bacteriological media until these were supplemented with a heat-labile factor and a heat-stable factor from blood. The naming of the heat-labile factor as a V or vitamin factor indicates that this work was being done in the context of animal vitamin studies.

The analytical approach, by the fractionation of complex culture media, was used in particular by J. H. Mueller (see J. H. Mueller, 1940) at Harvard, by the group led by Sir Paul Fildes (Medical Research Council Unit for bacterial chemistry, first at the London Hospital, 1929, and later at the Middlesex Hospital, 1934; see Knight, 1936, 1945), by André Lwoff and co-workers at the Institut Pasteur (see Lwoff, 1932, 1944) and other workers. Eventually numerous microbial growth factors were identified.

Wider implications of nutritional studies became evident when it was found that the same growth factors were essential nutrients for very different organisms, and that micro-organisms which did not require these substances as nutrients did indeed synthesize them. Cross-links with animal nutrition came with the finding that vitamin B_1, thiamine, was an essential nutrient for some micro-organisms. The recognition of an essential nutrient common to bacteria and animals stimulated work with bacteria, since these grew so much faster and were easier to handle. Later work established the reciprocal relation that some microbial growth factors were also animal vitamins.

The next step was the finding of functions for some of these animal vitamins and microbial growth factors as components of enzyme systems of general distribution in many kinds of organisms. It eventually became clear that the identification of essential nutrients was a way of finding parts of essential metabolic mechanisms commonly used by very different organisms, and that nutrient requirements reflected the abilities of different organisms to synthesize—in whole or in part—these components of generally used metabolic mechanisms (see Knight, 1936, 1945; Lwoff, 1932, 1944). It was to emphasize this that Fildes (1940) suggested the term 'essential metabolite' for use in place of the term 'growth factor', since the latter indicated a more limited status as a nutrient, while the term essential metabolite indicated a more general function as an essential component of the living cell. An essential nutrient for a given organism was an essential metabolite which that organism could not synthesize.

Biosynthesis and microbial genetics

Theories about the inter-relationship of nutritional requirements with ability in biosynthesis, based upon the comparative nutrient requirements of very different micro-organisms, and the possible relation of these differences to an evolutionary loss of ability to synthesize (Lwoff, 1932, 1944; Knight, 1936) were given a clear-cut basis by the work of Beadle and Tatum. If such losses in ability to synthesize were based on mutation and selection as required by modern concepts of evolution, then the capacities for the synthesis of essential metabolities in micro-organisms should be determined by genes, which should be subject to mutation as in other organisms. This is what Beadle & Tatum (1941) found in the successful production, by the use of mutagenic reagents, of nutritionally deficient or biochemical mutants of *Neurospora* sp. Biochemical mutants were later obtained from bacteria.

The way was thus opened for the study of biosynthetic and metabolic pathways by the use of micro-organisms blocked at selected steps in possible reaction chains. There is no need to emphasize how useful such mutants have been in the study of metabolic pathways. My predecessor D. D. Woods in the Inaugural Lecture (1953)

of this series discussed in detail the integration of research on the nutrition and metabolism of micro-organisms.

In the ways I have mentioned, from the 1920's on, it thus became possible to begin to study in detail how micro-organisms change the compounds of their environment into more of themselves. How they affect their environments, whether in an industrial process, in spoilage, in soil fertility, or in disease, are the results of their multiplication and the cellular metabolism which this involves.

When it was established that most characters of bacteria are controlled by hereditary units analogous to genes in classically sexual forms, wide possibilities began to emerge for the study of the mode of inheritance of characters. Such studies gave birth to the new field of microbial genetics which is now having such far-reaching developments. This work with micro-organisms has opened new perspectives in the study of biological inheritance and variation in general.

Microbiology

The physiological viewpoint in microbiological studies was clearly developed by Marjory Stephenson in the Introduction to her book *Bacterial Metabolism* (1930), and it remained a guiding principle through the rest of her life. How she visualized these physiological studies as contributing to our knowledge of the behaviour of micro-organisms in nature was summed up in the celebrated table of levels of research which she gave at the inaugural meeting of our Society in February 1945.

This table has often been mentioned; it was recorded briefly in an unsigned report of the inaugural meeting of the Society for General Microbiology published in *Nature* in February 1945, and in a modified form in D. D. Woods's Memorial Lecture in 1953. The actual table itself has not hitherto been published. I am glad to be able to show a picture of it today; it shows some interesting details not given in earlier references to it (Plate, fig. 1, and Appendix).

I am particularly glad to be able to show this table because it makes so clear the broad biological outlook of one whose life-work was mainly in the field of chemical microbiology. I hope I have not given the impression that I think that all microbiology is chemical microbiology. I do not; that is why I have often used the expression microbial physiology, meaning the functioning of the whole organism in its environment, which will include other organisms. This functioning is also expressed in biological terms, as in the questions of heredity, variation and competition.

You will see from this table (see Plate, fig. 1; and Appendix, p. 369) that the various levels of microbiological investigation, which Marjory Stephenson formally separated, include almost all biological aspects, at least by implication and extension, for which the most biological biologist could wish; for example, in the study of mixed cultures growing in natural environments. The time sequence indicated in the right-hand side of the table shows microbiology proceeding, in the left-hand column, to more and more detailed investigations, and more and more simplified systems. But one has only to study the content of this right-hand column to see that it embraces all aspects of the biological activity of the organisms. Furthermore, from the point of view of biology in general, one can remember that, because of their extremely wide range of physiologies, micro-organisms give one of the most fruitful and convenient fields for the study of that conception of the unity of biochemistry at the cellular level which Kluyver did so much to emphasize.

Marjory Stephenson's table sums up very well the attitude of the workers in many different fields of microbiology who came together to found our Society sixteen years ago, among whom she was one of the most far-sighted. In the minds of the founders of our Society there was a pretty clear idea of the need for attempting to weld the various parts of microbiology into a whole, or at least of helping to avoid the difficulties of communication between the different parts of microbiology which arise primarily from those 'eminent practical implications' which Kluyver remarked upon. In the last twenty years we have seen an enormous development of microbiology, especially of those fields concerned with bacteriophage, plant and animal viruses, microbial genetics, adaptive or inducible enzymes and the fine structure of cells.

Our Society has attempted to maintain a unified outlook on microbiology as the study of the organisms in their environments, how they multiply and how they may change. This is a central position from which microbial activities can be investigated and controlled. Our Society has tried to foster this unifying role by its meetings and through its *Journal*. The Symposia have had as objectives to bring together different kinds of work around selected topics, some narrower, some wider. Some of the topics have concerned rapidly developing fields, such as bacterial anatomy, viruses and microbial genetics. With other symposia the intention has been to survey wider fields, more slowly developing perhaps because more complex; for example the symposium on *Microbial Ecology* (1957) and to-morrow's symposium on *Microbial Reaction to Environment* (1961).

In the eighteenth century and the first half of the nineteenth century the means of scientific communication were relatively modest, and the difficulties of communication between different fields of microbiology seem to have been ideological, rather than practical. During the last fifty years the rate of publication has increased year by year, latterly at a daunting speed. Now the difficulty is not so much the lack or badness of communication but a plethora of print which tends to encourage splitting into highly specialized fields.

Our *Journal* tries to help in welding microbiology into a whole, or at least in keeping within one pair of covers a fairly catholic cross-section of what we think of as microbiology. This is not always easy. I think I might now make a plea as an editor that microbiologists should practise a certain continence in writing papers, should preserve a certain scientific chastity, as it were. To support this plea I would instance the relatively short list of papers which Marjory Stephenson wrote during nearly thirty years of work. Characteristically she did not publish until a piece of work had been well established and had reached a certain degree of completeness.

In conclusion I wish to show a picture (Plate, fig. 2) of the last paragraph of the abstract of the paper which Marjory Stephenson read at our Inaugural Meeting in 1945, because it shows in her own clear language her vision of the function of our Society:

> Unless work is to grow first stale and then sterile it must be refreshed by contacts with work at other levels. Moreover as knowledge increases and technique becomes more difficult interdepartmental collaboration is strongly indicated, a development which this society may do much to foster.

(See Appendix for the complete abstract.)

I hope we may all bear in mind the difficulties of this task of integration and also the great value of attempts to achieve it. This needs our continuous attention as new branches of microbiology continue to proliferate.

APPENDIX

Author's abstract of a paper read at the Inaugural Meeting of the Society for General Microbiology on 16 February 1945 at the London School of Hygiene and Tropical Medicine.

By Marjory Stephenson

The hopes which I build up around this Society can be summarized in two words, *contacts* and *motion*. At the outset I should like to confess that in the labelling of microbiological work I am somewhat of a heretic for I have come to believe that certain fashionable words are more mischievous than helpful. Pure science for example implies the existence of an impure variety whilst fundamental research suggests that someone else is occupied in superficial activities. Such terms are apt to introduce discord in the family, which is not one of the aims of our new society; I propose to banish them therefore from this discussion.

Levels of microbiological investigation

A	Mixed cultures growing in natural environments	Early Pasteur Period 1858—. Early studies on soil microbiology, and fermentation. Modern work on marine, river and lake populations, soil and ruminant digestion.
B	Pure growing cultures in laboratory media	Later Pasteur Period 1876—. Isolations in pure culture and the identification of the causal agents of many infectious diseases, specific fermentations and chemical changes in soil
C	Non-proliferating cells in pure culture on chemically defined substrates	Early studies (1919–) on intermediary processes of fermentation use of poisons, inhibitors and fixatives. Early enzymes studies—oxidations, reductions, deaminations, decarboxylations etc.
D	Pure growing cultures in highly purified media	Detailed study of growth requirements, microbial vitamins, nutritional antagonism, chemo-therapeutics, microbiological assay. 1930–
E	Cell-free enzymes and co-enzymes on pure substrates	Development of C. Greater precision in knowledge of fermentation processes and correlation with chemical activity of animal and plant tissues. Action of some growth factors as co-enzymes and of some soluble toxins as enzymes. 1940–

Nevertheless members here present work in many different fields or, as I have indicated in my diagram, at different levels which I have labelled A, B, C, D, E without any prejudice as to which is higher or lower still less which is fundamental or superficial. These levels are characterized by differences of technical approach. Level A is particularly characteristic of the early period of microbiology which began with the publication of Pasteur's first paper on fermentation in 1858. It comprises studies of mixed populations in natural media, and it will be recalled that much early work on fermentation, putrefaction and chemical changes in soil belongs here; but important modern work is done at this level, including the study of river, lake and marine populations, the enzymes of the soil and the chemical changes occurring in the rumen.

Level B involves the simplification of the biological material by the use of pure cultures; to this level belong the great triumphs of medical bacteriology where specific organisms were proved to be the causal agents of specific diseases or of chemical changes in soil or of well characterized fermentations; the greater part of medical and epidemiological bacteriology is still done at this level and almost every new discovery must start here. It is, however,

important to note the limitations of the method; you can by this technique attribute the overall effect to a given organism but you can learn little or nothing of the mechanism by which the effect is brought about. For example you can attribute a certain form of gas gangrene to a certain type of *Cl. welchii* but you can little of the mechanism by which the organism achieves the result.

At level C you proceed to the simplification of the substrate; you dissociate chemical activity from simultaneous growth and by the use of poisons and inhibitors you begin to unravel the mechanisms and stages by which the organism in pure culture produces its chemical effects. You can begin to study the cell as an association of enzyme systems. Neuberg's work on alcoholic fermentation was done at this level and the early work of the Cambridge School.

In E you proceed further and begin to separate the enzymes themselves and show how each produces its effect on its own particular substrate. Thus in the case of *Cl. welchii* (type A) the toxin or lethal factor has been partially purified and shown to be an enzyme, lecithinase, which achieves its dire results by disrupting the lecithin of the blood cells and tissues whilst the 'invasive factor' has been identified with hyaluronidase which by the destruction of hyaluronic acid facilitates the spread of the infective agent.

But it is not the filterable enzymes alone which can be separated and purified; several methods now exist for breaking up the bacterial cell. In the case of *Esch. coli*, for example, we have been able to extract several of the enzymes operating in the long chain of events by which the carbohydrate molecule is disrupted to the final stable products of fermentation. Examples of work at this level are flowing in fast and gathering in momentum and volume.

As we proceed from A to E first the biological agent then the substrate then again the biological agent are simplified till the process first discerned at B as cause and effect is finally shown to consist of an ordered march of complicated events.

But it is obvious how dependent on each other are the workers at different levels. Facts established at A and B provide the starting-point for work at C, D and E. But movement must be in both directions. For instance, the association of certain strains of Group D streptococci with epidemics of neo-natal diarrhoea have been tied up with the presence of the enzyme tyrosine decarboxylase; the ball was then tossed back to level B for epidemiological verification and animal experiment.

Unless work is to grow first stale and then sterile it must be refreshed by contacts with work at other levels. Moreover as knowledge increases and technique becomes more difficult interdepartmental collaboration is strongly indicated, a development which this society may do much to foster.

(*Signed*) MARJORY STEPHENSON

REFERENCES

AINSWORTH, G. C. (1951). A century of medical and veterinary mycology in Great Britain. *Trans. Brit. mycol. Soc.* **34**, 1.

BASSI, AGOSTINO (1835). *Del mal del segno*.... 1958: English translation by P. J. Yarrow, ed. G. C. Ainsworth, *Phytopath. Class.* (*Amer. phytopath. Soc.*), no. 10.

BEADLE, G. W. & TATUM, E. L. (1941). Genetic control of biochemical reactions in Neurospora. *Proc. nat. Acad. Sci., Wash.* **27**, 499.

BERKELEY, M. J. (1846). *Observations botanical and physiological on the potato murrain*.... 1948: *Phytopath. Class.* (*Amer. phytopath. Soc.*) no. 8. East Lansing, Michigan, U.S.A.

BOGHURST, WILLIAM (1666). *Loimographia*; an account of the great plague of London in the year 1665. 1894: now first printed from the British Museum Sloane MS. 349 for the Epidemiological Society of London. Edited by J. F. Payne. London: Shaw & Sons.

BRETONNEAU, P. F. (1826). *Des inflammations speciales du tissue musqueux et en particulier de la diphthérite*.... Paris: Crevot.

BROWN, J. C. (1913). *A History of Chemistry*. London: J. and A. Churchill.

BÜCHNER, E. (1897). Alcoholische Gärung ohne Hefezellen. *Ber.* **30**, 117.

BULLOCH, W. (1938, reprinted 1960). *The History of Bacteriology*. Oxford University Press.

CAGNIARD-LATOUR, C. (1837). Mémoire sur la fermentation vineuse. *C.R. Acad. Sci., Paris*, 4, 905.

CAGNIARD-LATOUR, C. (1838). Mémoire sur le fermentation vineuse. *Ann. Chim.* 68, 128.

DAVIS, T. L. (1945). Introduction to English translation by Huang Tza-Ch'ing and Chao Yun-ts'ung of: *The Preparation of Ferments and Wines* by Chia Ssu-Hsieh of the later Wei dynasty. *Harvard J. Asiat. Studies*, 9, 24.

DOBELL, C. (1932). *Antony van Leeuwenhoek and his 'Little Animals'*. London: John Bale, Son and Danielsson.

DOETSCH, R. N. (editor, 1960). *Microbiology: Historical Contributions from* 1776–1908. New Brunswick, New Jersey, U.S.A.: Rutgers University Press.

DUBOS, R. (1950). *Louis Pasteur: Free Lance of Science*. Boston: Little, Brown and Co. (with illustrations). [1951, London: Gollancz.]

DUCLAUX, E. (1896). *Pasteur: histoire d'un ésprit*. Sceaux: Imprimerie Charaire et Cie. 1920, English translation by Erwin F. Smith & Florence Hedges. Philadelphia and London: W. B. Saunders Company.

DUCLAUX, E. (1898–1902). *Traité de Microbiologie*. 4 tomes. Paris: Masson et Cie.

FILDES, P. & MCINTOSH, J. (1920). The aetiology of influenza. *Brit. J. exp. Path.* 1, 119.

FILDES, P. (1940). A rational approach to research in chemotherapy. *Lancet*, i, 955.

FRACASTORO, G. (1546). *De contagione*.... 1930: English translation by W. C. Wright. New York: G. P. Putnam's Sons. See Bulloch (1938), pp. 9–13.

GREEN, J. REYNOLDS (1899). *The Soluble Ferments and Fermentations*. Cambridge University Press.

GRUBY, D. (1842, 1843, 1844). See Ainsworth (1951); Bulloch (1935), pp. 165–6.

HARDEN, A. (1911). *Alcoholic Fermentation*. (1914, 2nd edition; 1923, 3rd edition; 1932, 4th edition.) London: Longmans, Green and Co.

HENLE, J. (1840). See Fildes & McIntosh (1920); Bulloch (1938), pp. 163–5.

JOBLOT, L. (1718). Descriptions et usages de plusieurs nouveaux microscopes tant simples que composez; avec de nouvelles observations faites sur une grand nombre d'insectes et sur d'autres animaux de diverses espèces qui naissent dans des liqueurs preparées et dans celles qui ne le sont point. Paris: Collombat. See Bulloch (1938), pp. 70–1.

KLUYVER, A. J. & VAN NIEL, C. B. (1956). *The Microbe's Contribution to Biology*. Cambridge, Massachusetts, U.S.A.; Harvard University Press.

KNIGHT, B. C. J. G. (1936). Bacterial nutrition; material for a comparative physiology of bacteria. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 210.

KNIGHT, B. C. J. G. (1945). Growth factors in microbiology; some wider aspects of nutritional studies with micro-organisms. *Vitam. & Horm.* 3, 108.

KOCH, R. (1878). *Untersuchungen über die Aetiologie der Wundenfectionskrankheiten*. Leipzig: Vogel. (1880): English translation by W. W. Cheyne: *Investigations into the Etiology of Traumatic Infective Diseases*, no. 88. London: New Sydenham Society. See Doetsch (1960).

KÜTZING, F. (1837). Microscopische Untersuchungen über die Hefe und Essigmutter nebst mehreren andern dazu gehörigen vegetabilischen Gebilden. *J. prakt. Chem.* 2, 385. See Bulloch (1938), pp. 50–3, 378.

LARGE, E. C. (1946). *The Advance of the Fungi*. London: Cape.

LAVOISIER, A. L. See, for example (1789). *Traité elementaire de chymie*, 2 tomes. Paris. See Brown (1913), pp. 288–9; Bulloch (1938), pp. 43–4.

LEEUWENHOEK (1676, 1683). See Dobell (1932).

LIEBIG, J. VON (1840). *Organic Chemistry in its Application to Agriculture and Physiology*. Ed. from MS. by Lyon Playfair. London: Taylor and Walter.

LIEBIG, J. VON (1870). *Ueber Gärung über Quelle der Muskelkraft und Ernährung*. Leipzig and Heidelberg: Winter.

LWOFF, A. (1932). *Recherches biochimiques sur la nutrition des protozoaires; le pouvoir de synthèse*. Paris: Masson et Cie.

LWOFF, A. (1944). *L'évolution physiologiques; études des pertes de fonctions chez les micro-organismes*. Paris: Hermann et Cie.

MUELLER, J. H. (1940). Nutrition of the diphtheria bacillus. *Bact. Rev.* 4, 97. See Knight (1945).

PASTEUR. The contribution of Pasteur to microbiology was, of course, cardinal; it has been much studied. Among outstanding books are those by Duclaux (1896), Bulloch (1935), Dubos (1950). Pasteur's *Études sur la bière* (1876) was published in English in 1879, translated by F. Faulkner and R. C. Robb (London: Macmillan). The collected publications and unpublished material have been collected in *Oeuvres de Pasteur* réunies par Pasteur Vallery-Radot (1933–39). Paris: Masson and Cie.

PASTEUR, L. (1857). Mémoire sur la fermentation appelée lactique. *C.M. Acad. Sci.* Paris, 45, 913. See Doetsch (1960).

PASTEUR, L. (1858). Sur la fermentation appelée lactique. *Ann. chim. Phys.* 3e ser. 52, 404.

PRÉVOST, BÉNÉDICT (1807). Memoir of the immediate cause of bunt or smut of wheat and of preventives of bunt. 1939: English translation by G. W. Keitt, *Phytopath. Class.* (*Amer. Phytopath. Soc.*), no. 6. Menasha, Wisconsin, U.S.A.

REDI, F. (1668). *Esperienze intorno alla generazione degl'insetti. Firenze.* English translation by M. Bigelow: *Experiments in the Generation of Insects.* Chicago: Open Court Publ. Co. See Bulloch (1938), pp. 67–70, 392.

SCHÖNLEIN, J. L. (1839). Zur Pathogenie der Impetigines. *Arch. Anat. Physiol., Lpz.* p. 82.

SCHWANN, T. (1837). 1847: English translation by H. Smith; *Microscopical Researches into the Accordance in the Structure and Growth of Animals and Plants.* London: Sydenham Society. Note on pp. 197–8 about yeast and fermentation. See Bulloch (1938), pp. 48–55, 296; Doetsch (1960).

SÉDILLOT, C. E. (1878). See Bulloch (1938), pp. 187, 397.

Society for General Microbiology (1945). Report of Inaugural Meeting. *Nature, Lond.* 155, 796.

Society for General Microbiology (1957). *Seventh Symposium: Microbial Ecology.* Edited by R. E. O. Williams & C. C. Spicer. Cambridge University Press.

Society for General Microbiology (1961). *Eleventh Symposium: Microbial Reaction to Environment.* Edited by G. G. Meynell & H. Gooder. Cambridge University Press.

SPALLANZANI, LAZZARO (1765; orig. edn.). See, Experiences pour servir a l'histoire de la generation des animaux et des plantes par M. l'Abbé Spallanzani, avec une ébauche de l'histoire des êtres organisés avant leur fecondation par Jean Senebier. 1785. Geneva: Barthelemi Chirol. See Bulloch (1938), pp. 75–80; Doetsch (1960).

STEPHENSON, M. (1930). *Bacterial Metabolism.* London: Longmans, Green and Co.

STEPHENSON, M. (1946). The debt of biochemistry to microbiology. Congrès des sciences pastoriennes. Pur le cinquantenaire de la mort de Louis Pasteur. Paris: 18–25 Novembre, 1946. Comité du Cinquantenaire; Union Nationale des Intellectuals.

TILLET, M. (1755). Dissertation on the cause of the corruption and smutting of the kernels of wheat in the head and on the means of prevention. 1937. English translation by H. B. Humphrey. *Phytopath. Class.* (*Amer. Phytopath. Soc.*), no. 5. Ithaca, N.Y., U.S.A.

TWORT, F. W. & INGRAM, G. L. Y. (1913). *A Monograph on Johne's Disease.* London: Baillière, Tindall and Cox.

TYNDALL, J. (1881). *The Floating Matter of the Air.* London: Longmans, Green. See *Life and Work of John Tyndall*, by A. S. Eve and C. H. Creasey, 1945, London: Macmillan; Doetsch (1960).

WOODS, D. D. (1953). The integration of research on the nutrition and metabolism of micro-organisms. The Inaugural Marjory Stephenson Memorial Lecture. *J. gen. Microbiol.* 9, 151.

aginag

(Delivered before the Society for General Microbiology at its Thirty-second Meeting, 10 April 1961)

	LEVELS OF MICROBIOLOGICAL INVESTIGATION	
A	mixed cultures growing in natural environments	EARLY PASTEUR PERIOD 1858 -- Early studies on soil microbiology, & fermentation. Modern work on marine, river & lake populations, soil & ruminant digestion.
B	Pure growing cultures in laboratory media.	LATER PASTEUR PERIOD 1876 -- Isolations in pure culture & the identification of the causal agents of many infectious diseases, specific fermentations & chemical changes in soil
C	Non-proliferating cells in pure culture on chemically defined substrates.	Early studies (1919 --) on intermediary processes of fermentation use of poisons, inhibitors & fixatives. Early enzymes studies -oxidations, reductions, deaminations, decarboxylations etc
D	Pure growing cultures in highly purified media	Detailed study of growth requirements microbial vitamins, nutritional antagonism, chemo-therapeutics, microbiological assay. 1930—
E	Cell-free enzymes & co-enzymes on pure substrates.	Development of C. greater precision in knowledge of fermentation processes & correlation with chemical activity of animal & plant tissues. Action of some growth factors as co-enzymes and of some soluble toxins as enzymes. 1940—

Fig. 1

Unless work is to grow first stale and then sterile it must be refreshed by contacts with work at other levels. Moreover as knowledge increases and technique becomes more difficult interdepartmental collaboration is strongly indicated, a development which this society may do much to foster.

Marjory Stephenson

Fig. 2

J. gen. Microbiol. (1962), **27**, 373–382
With 1 *Plate*
Printed in Great Britain

Genetic Recombination in the Hop-Wilt Fungus *Verticillium albo-atrum*

By A. C. HASTIE
Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 13 *October* 1960)

SUMMARY

Heterokaryons obtained between nutritional mutants of the imperfect fungus *Verticillium albo-atrum* (Reinke & Berth) were very unstable, and heterokaryotic hyphal tips growing out from mixed inocula were difficult to find. Heterokaryotic conidia were not detected, in spite of the fact that about 1 % of the spores contained two or three nuclei. Heterozygous nuclei in uninucleate conidia from mixed cultures yielded recombinant phenotypes at a higher frequency than usually observed in the other imperfect fungi which have been studied genetically. With no easily applicable criterion of chromosome number, such as spore size, the interpretation of results is difficult, but the genetic recombination demonstrated here seems likely to be accomplished through a parasexual system. Nuclei heterozygous at some loci but homozygous at others were formed from nuclei heterozygous at four marked loci, and were detected by isolating second-order segregants. These heterozygous segregants were presumably produced by mitotic recombination.

INTRODUCTION

The history of Verticillium wilt of hops follows a familiar pattern. The initial outbreaks reported by Harris (1927) were mild, but more virulent strains of the fungus were reported about 10 years later (Keyworth, 1939). Severe attacks by Verticillium have become more frequent and wilted plants of a hitherto wilt-tolerant variety were recently reported. It is therefore of interest to know whether genetic recombination may be a factor in the evolution of these more virulent strains. Genetic recombination has been reported in several fungi which, like *Verticillium albo-atrum*, have no known sexual stage (Pontecorvo, Roper & Forbes, 1953; Pontecorvo & Sermonti, 1954; Buxton 1956). Robinson, Larson & Walker (1957) suggested genetic recombination as a probable explanation of phenomena observed in their monoconidial cultures of *V. albo-atrum*, which appears to be a convenient subject for genetical investigations since the conidia are almost always uninucleate (Waggoner, 1956; Caroselli, 1957). The establishment of heterokaryons should present no difficulties, because Reinke & Berthold (1879) reported anastomosing germ tubes, and Caroselli (1957) showed that some of the hyphal cells contain several nuclei.

* Present address: Botany Dept., Queen's College, Dundee.

METHODS

Dark and hyaline variants of *Verticillium albo-atrum* (Reinke & Berth) isolated from wilted hop plants at East Malling Research Station, Kent, were used. The dark strains produced black torulose hyphae, not formed by the hyaline isolates. These were formed only irregularly on most media, but an augmented prune extract (PE) medium (Talboys, 1960) was found to be suitable for distinguishing between dark and hyaline strains.

Conidia of *Verticillium albo-atrum* are borne on verticillately branched conidiophores, and a drop of water containing up to thirty conidia is formed at the tip of each branch. Suspensions of conidia were readily prepared by flooding young slope cultures with a few millilitres of sterile distilled water. Hyphal fragments occurred only rarely in these suspensions, and the conidia did not clump as do those of dry-spored fungi when suspensions are prepared in this way. Thus it was reasonably certain that the colonies formed when aliquots of these suspensions were diluted and spread on agar media originated from single conidia. Isolations of single conidia were made at certain stages during the work. For this, a conidial suspension was spread on the surface of agar media and the conidia, which are about 7 μ long, were located under high magnification and isolated on small blocks of agar with the aid of a dissecting microscope. The nuclei in conidia were counted after staining with Giemsa. The staining schedule suggested by Hrushovetz (1956) was satisfactory, but shortening the hydrolysis time to 4 min. at 60° gave better results.

Stable nutritional mutants were obtained by exposing conidia to ultra-violet radiation supplied by a Hanovia XII medium-pressure lamp from which about 95 % of the radiation was emitted at 2537 Å. Conidia were exposed at 20 cm. from the ultraviolet source, at which distance the radiation intensity was about 108 μW/cm.^{2}. An exposure of 20 sec. gave only 3 % survivors, of which 0·5 % were nutritional mutants.

Wild type strains of the fungus grew rapidly on Czapek–Dox agar and this was used as a minimal medium. Complete medium (CM medium; Pontecorvo, 1953) was also extensively used, and the techniques of replication and auxanography described by Pontecorvo were used to characterize nutritional mutants. Heterokaryons were synthesized by mixing conidia of nutritional mutants, and streaking the mixed inoculum across the diameter of Petri dishes containing Czapek-Dox agar medium. Conidia containing heterozygous nuclei were selected by the technique of Roper (1952).

A spontaneous mutant resistant to acriflavine was selected by inoculating about 180×10^6 conidia on to CM medium containing 100 μg. acriflavine/ml. Acriflavine tolerance was determined on CM medium containing an appropriate concentration (300 μg./ml.) of acriflavine added to the agar medium immediately before it was poured into Petri dishes. This procedure gave consistent results despite the interaction between acriflavine and nucleic acid noted by McIlwain (1941), and there was no obvious lag phase during the early stages in growth of the resistant mutant.

The following symbols are used for convenient reference: *ad* = adenineless; *bi* = biotinless; *meth* = methionineless; *acr* = acriflavine-resistant.

RESULTS

Heterokaryosis

Conidia of a hyaline, methionineless and biotinless (*meth bi*) strain were mixed with conidia of a black adenineless (*ad*) isolate. The mixed inoculum, incubated on unaugmented Czapek–Dox agar on which neither strain can grow, produced fans of mycelium which outgrew the mixed inoculum, although growing more slowly than the wild type (Pl. 1, fig. 1). Most hyphal tips taken from these sectors failed to grow on Czapek–Dox agar medium, but eight hyphal tips from three sectors formed cultures which later yielded conidia of both parental types. Such cultures were clearly heterokaryotic. Because only parental type conidia were detected in samples of about 1000 conidia from each of these heterokaryons, it appeared that heterokaryotic conidia were produced very infrequently.

The intensity of the black phenotype varied more among colonies produced by 200 adenineless conidia from the heterokaryons than in a similar sample of conidia taken directly from the original pure culture. Because of this variability the mycelial colour will not be mentioned in the section on genetic recombination but will be considered further in the discussion.

Selection of prototrophic conidia

A spontaneous mutant resistant to acriflavine was selected from the methionineless and biotinless strain used to investigate heterokaryosis. This further marker gave the strain the phenotype *acr meth bi*. Conidia from this strain were mixed with conidia from the black adenineless acriflavine-sensitive isolate previously used, and the mixed inoculum was incubated on slopes of Czapek–Dox agar medium. Similar samples of conidia from these mixed cultures were spread on Czapek–Dox, and

Table 1. *Numbers of prototrophic conidia recovered from five heterokaryons of* Venticillium albo-atrum

Heterokaryotic mycelium was selected by incubating two auxotrophic strains on Czapek–Dox agar (*acr meth bi* × *ad*). Conidial suspensions prepared from these heterokaryons were then spread on Czapek–Dox agar and Czapek–Dox + acriflavine to select prototrophs and acriflavine-resistant prototrophs, respectively.

Heterokaryons	No. of conidia tested on each medium ($\times 10^6$)	Media	
		Czapek–Dox	Czapek–Dox + Acriflavine
CF1	10·8	3	0
CF2	12·5	8	0
CF4	18·3	9	0
CF5	16·4	10	0
CF10	10·5	3	0
Total	68·5	33	0

Czapek–Dox containing 100 μg./ml. acriflavine, and the number of colonies formed after 4 days' incubation are recorded in Table 1. The recovery rate was approximately one prototrophic conidium per two million tested, and the test also indicated that these prototrophic conidia were acriflavine-sensitive. To confirm that the prototrophic propagules were uninucleate conidia, suspensions of conidia were

prepared from four of the colonies recorded in Table 1, and aliquots of these suspensions stained with Giemsa. About 99 % of the conidia in each of these four suspensions were uninucleate, and of about 4000 conidia examined none had more than three nuclei. Other samples of the same suspensions were spread at low density on both CM medium and Czapek–Dox medium to estimate the proportion of prototrophs. None of the four suspensions contained less than 30 % prototrophic conidia. Because there were very few, if any, non-viable conidia it must be concluded that almost all the prototrophic conidia were uninucleate. The analysis of heterokaryons indicated that multinucleate conidia must be homokaryotic.

Recovery of recombinant phenotypes

Single conidia isolated from one of the suspensions referred to above were incubated on slopes of Czapek–Dox medium, and only the prototrophs retained. Conidia from the resulting cultures harvested after incubation for 10 days were spread on CM medium at a density of about thirty per Petri dish. The colonies formed were re-isolated in a regular pattern for replication on an approximate series of test media (Pl. 1, fig. 2), and the phenotypes of about 100 isolates from each of twenty monoconidial cultures are given in Table 2. It is quite certain that each of the twenty cultures analysed was produced by a single conidium, which was likely to be uninucleate or at least homokaryotic, and the phenotypes recovered show that these conidia must have contained nuclei heterozygous for the four markers. There are therefore sixteen possible phenotypes obtainable from each culture, and in Table 2 these are arranged in reciprocal pairs.

The frequencies with which any two phenotypes were recovered can be compared by estimating the mean difference in the numbers of observations over the twenty replicates, and obtaining a 't' test from the ratio of this mean difference to its standard error. The parental phenotypes *acr meth bi* +, and + + +*ad* were recovered most frequently, and the recovery rates did not differ significantly ($P = 0{\cdot}3$ to $0{\cdot}2$). This is also true of the reciprocal phenotypes *acr* + *bi ad* and + *meth* + + ($P = 0{\cdot}3$ to $0{\cdot}2$), and it can similarly be shown that each of these pairs was recovered in significantly different numbers ($P < 0{\cdot}001$). There is therefore a tendency for reciprocal pairs to be recovered equally frequently although the phenotypes *acr* + + + and + *meth bi ad* are an obvious exception to this since 469 of the former were found, but only one of the latter. This difference can be accounted for by clonal multiplication of the original heterozygous nucleus in each culture.

The isolation of second-order segregants

Conidial dimensions provide a convenient distinction between haploid and diploid recombinants in some imperfect fungi shown to have a parasexual genetic system (Pontecorvo, 1956). Unfortunately the frequency distribution of the conidial dimensions in cultures of the parental strains used here did not differ significantly from other cultures known to have originated from a single heterozygous conidium.

The twenty cultures analysed in Table 2 were produced by conidia containing nuclei heterozygous at all four loci, and if the segregants were formed by parasexual mechanisms (haploidization and mitotic recombination) we should expect some of the first-order isolates recorded in Table 2 to be haploid and the others diploid.

Table 2. *Phenotypes of random isolates from twenty independent cultures formed by heterozygous conidia of* Verticillium albo-atrum

The heterozygous strain was selected from the heterokaryon *acr meth bi* × *ad*, and the random isolates were obtained by spreading conidial suspensions from the twenty heterozygous cultures on CM.

	Phenotype	Independent cultures (replicates)																				Tot
1	*acr meth bi*+	31	4	15	11	42	25	42	27	46	20	25	29	23	2	16	30	47	32	45	3	51
2	+ + +*ad*	34	56	24	29	29	40	37	35	36	38	19	24	18	1	23	36	30	46	37	9	58
	acr +*bi*+	2	4	4	—	—	1	—	5	—	4	1	—	7	1	2	4	4	7	1	—	4
	+*meth*+*ad*	—	—	—	—	4	1	—	—	—	4	—	—	—	—	1	2	—	—	—	—	1
	acr meth + +	—	1	2	2	—	2	—	—	—	6	1	1	1	2	5	2	2	1	—	6	3
	+ +*bi ad*	1	1	—	—	—	—	—	—	—	—	1	—	—	2	—	—	—	—	—	—	
	acr + +*ad*	—	6	—	15	—	—	—	—	—	—	3	—	3	—	1	5	1	2	—	1	3
	+*meth bi*+	—	—	—	1	—	—	2	—	—	—	—	3	—	—	2	—	—	—	—	—	
	acr +*bi ad*	3	—	5	29	4	1	1	2	6	2	—	3	3	20	1	8	5	4	7	17	12
	+ *meth* + +	2	—	1	3	6	4	4	2	4	—	4	—	1	23	5	3	1	2	2	12	7
3	*acr* + + +	31	25	44	12	9	20	8	28	11	30	43	29	42	48	46	8	12	6	7	25	46
	+*meth bi ad*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	
	acr meth bi ad	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	1	2	
	+ + + +	—	—	2	—	—	4	2	2	—	—	2	6	—	—	—	6	1	1	—	6	3
	acr meth+*ad*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	+ +*bi*+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total	104	102	97	102	94	98	96	101	103	104	99	95	98	101	102	104	103	101	100	82	193

1 and 2, parental phenotypes. 3, phenotype of original heterozygotes as determined by replication.

The diploid first-order segregants would be homozygous for some markers, and heterozygous at the other loci, and would therefore be detected by their capacity to produce further segregant phenotypes (second-order segregants), as shown in *Aspergillus nidulans* by Pontecorvo, Tarr-Gloor & Forbes (1954).

The very high yield of recombinant phenotypes from the 10-day-old 'mosaic' cultures, segregating at all four loci, suggested that heterozygous first-order segregant genotypes may be readily detected in random samples of conidia from these cultures, but they were not. However, when 'mosaic' cultures, segregating at all four loci, were grown on an augmented prune extract medium, the colonies were slightly slower growing and more fluffy than pure cultures. Thus it seems that second-order segregants may be recovered from colonies with this morphology which also have nutritional deficiency or are sensitive to acriflavine (i.e. fluffy first-order segregants). Four colonies with these characters were noticed during the later stages of the work, and two of them are shown in Pl. 1, figs. 3 and 4. Mono-conidial isolates were made from each of these and Table 3 gives the classification of approximately 100 isolates from each purified culture. Plate 1, fig. 5, shows the phenotypes recovered in a sample of twenty-six random isolates from a heterozygous segregant culture.

Table 3. *Phenotypes of second-order isolates from four heterozygous segregant cultures of* Verticillium albo-atrum

The heterozygous segregants were recovered amongst random first-order isolates from the heterozygote *acr meth bi/ad*, and their probable genotypes inferred from the characterization of second-order isolates.

Phenotypes of second-order isolates	Phenotypes of the heterozygous segregant cultures			
	++++	++++	++++	*acr meth*++
++++	33	19	75	—
+*meth*++	24	39	2	48
+++*ad*	39	43	9	—
+*meth*+*ad*	—	3	—	—
+*meth bi*+	—	—	10	6
++*bi*+*	—	—	4	—
acr meth bi+	—	—	—	36
acr meth ++	—	—	—	10
Probable genotypes of the original heterozygous segregant nuclei	+*meth*++ +++*ad*	+*meth*++ +++*ad*	+*meth bi*+ +++*ad*	*acr meth bi*+ +*meth*++

* This phenotype was not observed among random first-order isolates (Table 2).

The four loci segregated at approximately the same frequency in the cultures reported in Table 2 and if the conidia which initiated the cultures recorded in Table 3 also contained nuclei heterozygous at all four loci we should again expect all loci to show similar segregation rates. The column on the left of Table 3 shows that culture to be segregating at only two loci (*meth* and *ad*), and the proportion of non-segregants at each of these loci is approximately 2/3. This means that the probability of any single conidium taken at random from this culture being, say, methionine independent is 2/3. The chance of getting ninety-six *meth*+ spores from this culture in that many trials is then $(2/3)^{96}$. As the biotin locus can be expected to segregate at a similar frequency when it is heterozygous, the probability of the

original nucleus being heterozygous *bi*/+ is therefore about $(2/3)^{98}$, and there is even less chance of the nucleus being heterozygous at both the acriflavine and biotin loci. This reasoning has also been applied to the other three cultures recorded in Table 3, and the genotypes suggested by the observed segregations are given at the bottom of that Table. The single conidia from which the cultures were grown could not have been merely heterokaryotic, rather than heterozygous, as the isolates made from each culture included a high proportion which were fluffy like the original culture, had the same phenotype, and which showed similar segregation patterns.

Attempts to select rare acriflavine-resistant recombinants from the sensitives shown in Table 3 failed. This gives further support to the suggestion that the original conidia contained nuclei homozygous, or hemizygous, at the non-segregating loci. The genotypes suggested for these nuclei at the bottom of Table 3 assume they were diploid but there is no proof of this in the absence of a convenient criterion of chromosome number. Loci showing segregation must be heterozygous, but other loci in the same nucleus which do not segregate may be either haploid (hemizygous) or homozygous and diploid. These nuclei could arise by either the loss of one or more chromosomes from the diploid set, or by mitotic crossing-over, and there is no way of distinguishing between these possibilities using only the present data.

DISCUSSION

Variation in wild-type cultures of *Verticillium albo-atrum* has been described by Waggoner (1956), Caroselli (1957), Robinson *et al.* (1957), and Van den Ende (1958). Their cultures usually produced black torulose hyphae but variants apparently unable to do so were often observed. All forty single conidia which Robinson *et al.* (1957) took from a hyaline monospore culture produced cultures with black hyphae, and they suggested this was probably a result of genetic recombination. It seems at least equally likely that the original hyaline culture was potentially black (i.e. had the appropriate genotype) but failed to develop this phenotype; environmental factors such as pH, temperature, carbohydrate source, presence of alcohols, and even the depth of the medium, can all greatly influence the development and colour of these hyphae.

The doubly auxotrophic parent (*acr meth bi*+) used in my work never formed black hyphae on any of the media used, but the adenineless parent always did to some extent when grown on augmented prune extract. The analysis of heterokaryons between these strains did not convincingly demonstrate that the black character was entirely controlled by the nucleus. Further, nuclei heterozygous for all the selected markers always formed colonies with black hyphae, and no isolate from these colonies had the hyaline phenotype of the respective parent, although a few recombinants were obtained which formed black hyphae only rarely. The existing evidence suggests that cytoplasmic factors have a dominant role in the expression, and perhaps the inheritance, of the mycelial colour. Colour was certainly not inherited as a single gene difference otherwise it would have segregated at a similar rate to the nutritional markers. There is certainly no reason to suppose that the hyaline variants often isolated from wild-type cultures of *Verticillium albo-atrum* always arise by genetic recombination.

The most striking feature of the genetic recombination reported here is the rela-

tively high frequency at which it occurs. No attempt was made to estimate the absolute frequency with which recombinant phenotypes were produced, but it must certainly be greater than that usually observed in the other imperfect fungi previously studied (Pontecorvo *et al.* 1953; Pontecorvo & Sermonti, 1954; Buxton, 1956). Relatively high rates of recombination induced by mutagens have been reported in *Aspergillus orzyae* (Ikeda, Ishitani & Nakamura, 1956), *Penicillium chrysogenum* (Morpurgo & Sermonti, 1959), and *Aspergillus nidulans* (Käfer, 1960).

The high frequency of recombination leads to an anomaly seen by comparing Tables 1 and 2. The results in Table 1 indicate that the heterozygous prototrophic conidia selected directly from the heterokaryon were acriflavine-sensitive, but heterozygotes of the same genotype were classified as acriflavine-resistant in Table 2 for which the conidia were characterized by replication as opposed to direct-plating (Table 1). By replication we seek to classify a conidium by replicating the colony which it produces, and therein assume a relatively pure culture was formed. The discrepancy between the results in Tables 1 and 2 may be resolved by assuming acriflavine-resistance is recessive to sensitivity. Single heterozygous conidia (*acr*/+) tested by direct-plating will then be acriflavine sensitive (Table 1), but because the novel genotypes *acr* and *acr*/*acr* arise frequently during growth of a colony they may be transferred to the test medium in replicating, and the isolate will consequently appear acriflavine-resistant although it was in fact formed by an acriflavine-sensitive conidium. Corresponding misclassification cannot arise with respect to the nutritional markers, and the high frequency of segregation and recombination remains even if the acriflavine marker is ignored.

The genetic system in *Verticillium albo-atrum* seems likely to be mitotic rather than meiotic, and the question arises whether the high rate of recombination reported here is usual in *V. albo-atrum* or results from an irregular chromosome complement in the heterozygote. Analysis of the colonies produced by thirty-three prototrophic conidia selected directly from eight mixed cultures of the two parental strains gave results similar to those recorded in Table 2, and this regularity suggests that the very unstable heterozygous nuclei are those formed initially by nuclear fusions. If one of the parent strains had a disomic chromosome number (say $n+1$), selection within this strain should be expected to favour a haploid genotype, and heterozygous diploid nuclei rather than trisomics (say $2n+1$) would usually be formed in mixed cultures by nuclear fusions between the complementary parental genotypes. It is therefore unlikely that one parent strain with an irregular chromosome number would consistently lead to the production of heterozygotes with a correspondingly abnormal chromosome number, and that this could cause the high frequency of recombination.

A chromosomal rearrangement, or structural change, involving a centromere in one of the parents, so that non-disjunction or loss of one chromosome is frequent, could explain the results obtained. Loss of one chromosome from some nuclei in a haploid strain would go undetected because the nullisomic nuclei formed would be eliminated, but if the same chromosome was lost from a diploid nucleus this would result in monosomic nuclei which would not be eliminated, but tend to give further segregation. Before deciding whether the high frequency of recombination observed here is usual in *Verticillium albo-atrum*, more heterozygotes from independently isolated wild-type cultures will have to be analysed.

In Table 2 there is a tendency for the pairs of reciprocal phenotypes to be recovered equally frequently, and each pair at a significantly different rate with the parental classes most frequent. This pattern clearly suggests the four markers are linked, but elucidation of the formal genetics of this heterozygote requires that the haploid genotypes included in the data in Table 2 be considered separately.

The work described in this paper was done during the tenure of a Research Studentship awarded by the Agricultural Research Council (A.R.C.). I thank the A.R.C. for their support, Dr E. W. Buxton, Rothamsted Experimental Station, for his helpful suggestions, and Professor G. Pontecorvo, F.R.S., University of Glasgow, for his criticisms of the original draft. I also wish to acknowledge my thanks to Mr F. Cowland, Rothamsted Experimental Station, for taking the photographs.

REFERENCES

BUXTON, A. W. (1956). Heterokaryosis and parasexual recombination in pathogenic strains of *Fusarium oxysporum*. *J. gen. Microbiol.* **15**, 133.

CAROSELLI, N. E. (1957). Verticillium wilt of maple. *Bull. R. I. agric. Exp. Sta.* 335.

HARRIS, R. V. (1927). A wilt disease of hops. *Rep. E. Malling Res. Sta. for* 1925.

HRUSHOVETZ, S. B. (1956). Cytological studies in *Helminthosporium sativum*. *Canad. J. Bot.* **34**, 321.

IKEDA, Y., ISHITANI, C. & NAKAMURA, K. (1956). A high frequency of heterozygous diploids and somatic recombination induced in imperfect fungi by ultra violet light. *Proc. int. Genet. Symp.* p. 547.

KÄFER, E. (1960). High frequency of spontaneous and induced somatic segregation in *Aspergillus nidulans*. *Nature, Lond.* **186**, 619.

KEYWORTH, W. G. (1939). Verticillium wilt of hops. *Rep. E. Malling Res. Sta. for* 1938, 244.

MCILWAIN, H. (1941). A nutritional investigation of the anti-bacterial action of acriflavine. *Biochem. J.* **35**, 1311.

MORPURGO, G. & SERMONTI, G. (1959). Chemically induced instability in a heterozygous diploid of *Penicillium chrysogenum*. *Genetics*, **44**, 137.

PONTECORVO, G. (1953). The genetics of *Aspergillus nidulans*. *Advanc. Genet.* **5**, 141.

PONTECORVO, G. (1956). The parasexual cycle in fungi. *Annu. Rev. Microbiol.* **10**, 393.

PONTECORVO, G., ROPER, J. A. & FORBES, E. (1953). Genetic recombination without sexual reproduction in *Aspergillus niger*. *J. gen. Microbiol.* **8**, 198.

PONTECORVO, G. & SERMONTI, G. (1954). Parasexual recombination in *Penicillium chrysogenum*. *J. gen. Microbiol.* **11**, 94.

PONTECORVO, G., TARR-GLOOR, E. & FORBES, E. (1954). Analysis of mitotic recombination in *Aspergillus nidulans*. *J. Genet.* **52**, 226.

REINKE, J. & BERTHOLD, G. (1879). Die Zersetzung der Kartoffel durch Pilze. *Untersuchungen aus dem Botanischen Laboratorium der Universität Göttingen*, **1**, 1.

ROBINSON, D. B., LARSON, R. H. & WALKER, J. C. (1957). Verticillium wilt of potato in relation to epidemiology, symptoms and variability of the pathogen. *Univ. Wis. Res. Bull.* p. 202.

ROPER, J. A. (1952). Production of heterozygous diploids in filamentous fungi. *Experimentia*, **8**, 14.

TALBOYS, P. W. (1960). A culture-medium aiding the identification of *Verticillium albo-atrum* and *V. Dahliae*. *Plant Path.* **9**, 57.

VAN DEN ENDE, G. (1958). Untersuchungen über den Pflanzenparasiten *Verticillium albo-atrum*. *Acta bot. neerl.* **7**, 665.

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

EXPLANATION OF PLATE

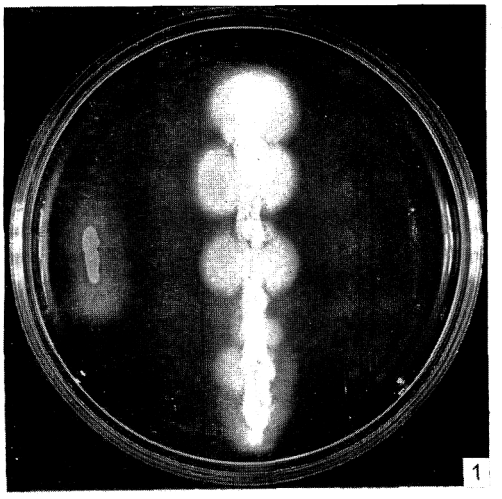
Fig. 1. Pure and mixed inocula of auxotrophs on Czapek–Dox agar. Adenineless strain (left), methionineless and biotinless strain (right), and mixed inoculum producing heterokaryotic sectors (centre).

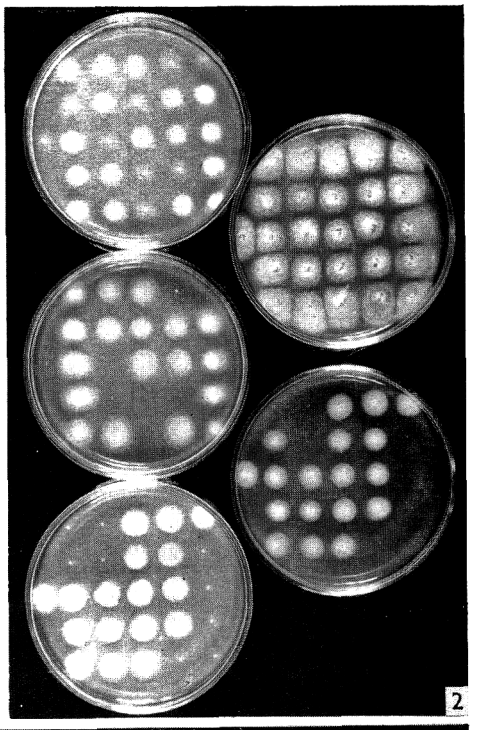
Fig. 2. Random monoconidial isolates from a heterozygous monoconidial culture. Template cultures grown on PE medium (top right) and replicates of this template on CM medium containing 300 μg/ml. acriflavine (lower right), biotinless medium (top left), methionineless medium (mid-left), and adenineless medium (bottom left) ($\times \frac{1}{2}$).

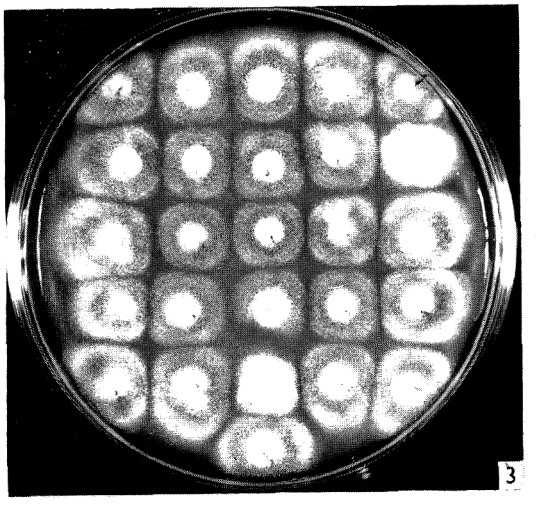
Fig. 3. Two fluffy (heterozygous) isolates in twenty-six random monoconidial isolates from a segregating culture (PE medium).

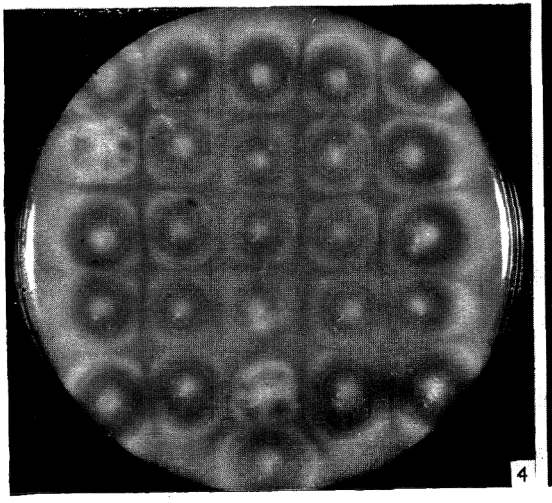
Fig. 4. Reverse view of the isolates shown in Fig. 3 to show sectoring associated with fluffy phenotypes (PE medium).

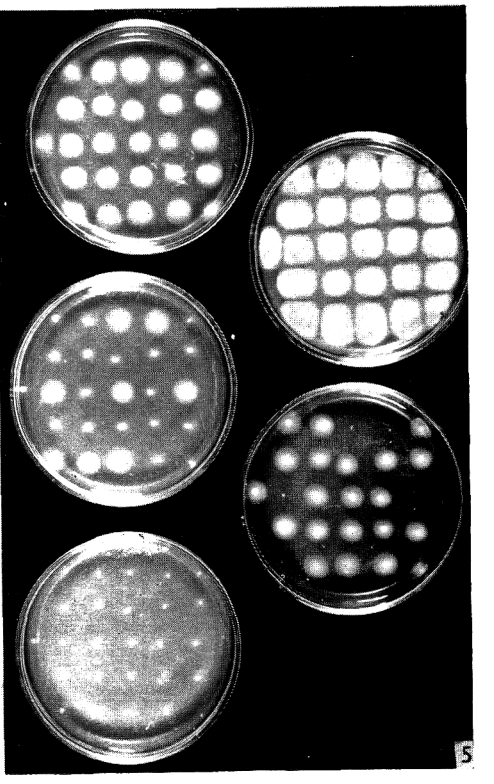
Fig. 5. Random monoconidial isolates from a heterozygous monoconidial segregant culture. Template cultures grown on PE medium (top right), and replicates of this template on CM medium containing 300 μg/ml. acriflavine (lower right), methionineless medium (top left), biotinless medium (mid left), and adenineless medium (bottom left) ($\times \frac{1}{2}$).











A. C. HASTIE

(*Facing p.* 382)

J. gen. Microbiol. (1962), **27**, 383–389
With 2 plates
Printed in Great Britain

Observations on Corynebacteria and Related Pleuropneumonia-like Organisms (PPLO)

By PHYLLIS PEASE and NANCY LAUGHTON
Department of Bacteriology, University of Birmingham

(*Received* 28 *December* 1960)

SUMMARY

Morphological, cultural and serological evidence is presented to suggest that a relationship exists between corynebacteria of human vaginal origin and pleuropneumonia-like organisms (PPLO). It is concluded that many PPLO are L-forms of corynebacteria, and that the differences between these and the L-forms of Gram-negative bacteria, most commonly studied, are in conformity with the differences between the parent bacterial forms.

INTRODUCTION

Numerous workers have reported an association between pleuropneumonia-like organisms (PPLO) and strains of corynebacteria (Minck, 1953; Wittler, Cary & Lindberg, 1956; Smith, Peoples & Morton, 1957), and have adduced evidence that the former may become transformed into the latter. The PPLO studied by Wittler *et al.* was found to be serologically related to a corynebacterium isolated from the same source, the human genital tract. The present study was initiated as a result of observations made on two types of colony, which arose in the same cultures of a corynebacterium isolated from a pathological condition; one was bacterial and the other resembled a PPLO. Each was capable of transformation into the other, and they appeared, in fact, to be different phases of the same organism. These transformations occurred, regularly but infrequently, in the course of investigations concerned with their pathogenicity, which involved their artificial culture on solid and in liquid medium and in embryonated eggs. The PPLO was identified as such because of its morphological, cultural and serological characters (see Discussion for references). The bacterium closely resembled the organism isolated from the human *cervix uteri* and named *Corynebacterium cervicis* (Laughton, 1951, 1954). In an attempt to elucidate the relationship between them, observations were made upon both these forms, and upon human genital tract PPLO isolated in this and other laboratories.

METHODS

Organisms. The first corynebacterium and the PPLO were obtained, by the courtesy of Dr K. B. Rogers, as primary isolation cultures taken at post-mortem from the lungs of a baby showing the condition known as hyaline membrane; the cultures consisted of a chocolate agar plate bearing both types of colony, and a blood plate with the PPLO colonies only. This original strain of the corynebacterium and those subsequently derived from the PPLO were identified as *Corynebacterium cervicis* (Laughton, 1951, 1954) by their morphology, cultural reactions

and serological reactions. Other PPLO isolated in this laboratory were obtained by the direct inoculation of Difco PPLO medium and chocolate agar with vaginal swabs taken at a Birmingham V.D. clinic by Miss D. M. Shotton. In cases where the plates were heavily contaminated with other organisms, a piece of filter paper, soaked in a solution containing 200 units penicillin/ml., was placed on the medium subsequently used to obtain pure cultures of PPLO. One PPLO was obtained by dissociation from a strain of corynebacterium also isolated from a vaginal swab. Four strains of *Mycoplasma hominis* Type 1 (H50R, H26, H23, D419) and one of *M. hominis* Type 2 (strain Campo originally isolated by Dr Dienes) were obtained by courtesy of Dr D. G. ff. Edward (Wellcome Research Laboratories, Beckenham) and used for purposes of comparison.

Cultural methods. Embryonated eggs were inoculated via the yolk sac and the intravenous and amniotic routes. All inocula were checked for purity and viability by plating concurrently upon artificial media. Control eggs in each series were inoculated with the corresponding suspension fluid without organisms. Media used were Difco PPLO medium and chocolate agar, and Hartley's broth with 10 % (v/v) human plasma or horse serum. Fermentation reactions of the PPLO were tested in a blood broth (made by adding 2 %, v/v, horse blood cells and 0·5 %, w/v, oxoid yeast extract to nutrient broth, steaming, filtering, adjusting to pH 7·8 and then autoclaving) with 20 % (v/v) de-activated horse serum and 1·0 % (w/v) carbohydrate; the pH values were taken by means of a capillator after 1, 2 and 3 weeks. The fermentation reactions of the corynebacteria were tested in serum water sugar media.

Serology. Antisera were prepared in rabbits by the intravenous injection of suspensions made up to an opacity of a No. 10 Brown's opacity tube of bacteria, and of PPLO in 0·2 % (v/v) formal saline. Five weekly courses of three 2 ml. doses were given. Some preliminary complement-fixation tests were performed, but these were subsequently abandoned in favour of gel diffusion tests, which proved to be more convenient and informative. To prepare the bacterial antigen for the complement-fixation tests, suspensions of the bacteria were made in 0·5 ml. of 0·01 N-sodium carbonate, 0·05 g. trypsin (Difco) added, and the mixture incubated for 18 hr. at 37°, after which 1 ml. of acid ethanol (95 vol. absolute ethanol + 5 vol. 2 N-hydrochloric acid) was added. The mixture was centrifuged and the deposit washed several times in saline to neutralize it. The deposit was then made up to an opacity of a No. 10 Brown's opacity tube. The PPLO antigen for the complement-fixation tests consisted of saline suspension made up to an opacity of a No. 2 Brown's opacity tube. Complement was titrated before each test and each test was set up in triplicate, with a suitable range of dilutions, usually 2, 2·5 and 3 minimal haemolytic doses. All appropriate controls were set up in each test. The antigens used for the gel diffusion tests consisted of bacterial and PPLO saline suspensions of four times the strength of those used for the production of antisera and, in some cases, were supersonically disintegrated. The tests were made by double diffusion precipitation in agar/phosphate gel at pH 7·4. To avoid false cross-reactions, bacteria for serum production were grown on glucose agar, and PPLO on media with horse serum. For test antigens, PPLO were grown on media with human plasma.

Electron microscope examinations. The electron microscope used was a Metropolitan Vickers E.M. 3, and all the preparations were gold + palladium shadowed.

The organisms except for the transition stages were grown upon collodion membranes for various periods of time before mounting. The preparations of the transition stages were obtained by impressing agar blocks containing the transforming organisms on to collodion membranes. Gentle pressure was applied, and the blocks were removed 3–4 hr. later. The membranes were then mounted.

RESULTS

In the early observations, two types of colony, the bacterial and the PPLO from the original cultures, were subcultured on chocolate agar and thence into Robertson's meat broth. On chocolate agar, both types of colony grew unchanged in subcultures; but subcultures on chocolate agar from the meat broth cultures inoculated with the PPLO alone, after these had been stored for 6 weeks at 4°, gave evidence of transformation to the bacterial phase. When replated upon chocolate agar these cultures gave rise to colonies both of the PPLO and of a corynebacterium resembling the original strain. The cultures inoculated with the bacteria alone showed only bacterial colonies on replating on this occasion but subsequently these were also observed, after the same procedure, to give rise to PPLO colonies; and on repeated subculture on chocolate agar from chocolate agar, over an extended period of time, the bacterial colonies gave rise to both types on approximately twelve occasions, and to PPLO colonies alone on one.

Transformation of the PPLO to the corynebacterium was observed, in artificial culture, only after subculture in fluid medium; this occurred on two occasions, after the lapse of a year from the time of the first observations, during which time the PPLO had been subcultured on chocolate agar, at intervals of from ten to fourteen days, without any sign of reversion. On one occasion the transformation was occasioned, as before, by storage in meat broth at 4° for several weeks, on the other simply by subculture in liquid yeast-extract medium. On both occasions, subculture on chocolate agar gave both types of colony.

In order to eliminate any question of contamination, repeated efforts were made, without success, to isolate similar PPLO from the mouths and noses of the operators and their associates.

It was discovered that these transformations occurred more frequently on culture in embryonated eggs than on artificial media; the results of these experiments are shown in Table 1. Cultures from control eggs showed no sign of any comparable organisms.

The results of fermentation tests showed that the PPLO did not ferment any of the carbohydrates examined, nor did 3 out of the 6 strains of the corynebacterium tested; a further 3 strains of the corynebacterium were found to ferment, irregularly, glucose, lactose, sucrose, dextrin, maltose, and starch, but not mannitol, dulcitol or salicin.

The results of complement-fixation tests are summarized in Table 2. The antigens used were the PPLO and corynebacterium as first isolated, and variants of *Corynebacterium cervicis* maintained in this laboratory. The sera used were those prepared against the original corynebacterium and PPLO, and the '9 T' variant of *C. cervicis*. The results show a relationship between the PPLO and both strains of corynebacterium. A control serum, prepared against a species of actinomyces, gave negative reactions.

At a considerably later date, a strain of corynebacterium, resembling but not identical with, *Corynebacterium cervicis*, also from the vagina, when grown in the vicinity of 200 units penicillin/ml., absorbed on a strip of filter paper on the surface of Difco PPLO medium with 3·5 % added sodium chloride, was found regularly to dissociate and produce an L-form or PPLO which was serologically related to the other PPLO examined (Table 3). This PPLO was unstable, except in the presence of penicillin, and reverted readily to the bacillary form.

Table 1. *Results of cultivation in embryonated eggs*

The eggs were 5–6 days old for the yolk sac, and 10–12 days for other routes. Subcultures were made after 1 to 4 days, according to the viability of the embryo.

Inoculum and no. of eggs	Route of inoculation	Material subcultured	Organisms recovered on subculture
Corynebacterium 9	Yolk sac	Yolk	1 PPLO only 2 PPLO and corynebacterium 6 corynebacterium only
Corynebacterium 6	Intravenous	Lung, liver amniotic fluid	1 PPLO and corynebacterium 1 no organisms recovered 4 corynebacterium only
Corynebacterium 6	Amniotic	Tracheal washings and lung	6 corynebacterium only
PPLO 6	Yolk sac	Yolk	6 PPLO only
PPLO 19	Amniotic	Tracheal washings and lung	18 PPLO only 1 PPLO and corynebacterium

The results of gel diffusion tests on all these strains are shown in Table 3. The antigens were the original corynebacterium, after 2 years' subculture, the original PPLO (0), PPLO (1, 2, 3, 4, 5) isolated in this laboratory, and 5 strains of human PPLO supplied by Dr D. G. ff. Edward. The sera were prepared against the original PPLO (0); one of those isolated in this laboratory, PPLO (1); and the penicillin-induced dissociant, PPLO (P). The results given by this method were very clear-cut,

Table 2. *Complement-fixation tests*

	Antiserum		
Antigen	PPLO phase	Bacterial phase	*Corynebacterium cervicis* 9, 'T'
PPLO phase	4	3	3
Bacterial phase	1	4	1
C. cervicis 9, 'O'	3	3	4
C. cervicis 9, 'T'	3	4	3

Figures indicate degrees of reaction.

and showed a serological relationship between most of the PPLO tested, whether these were associated with corynebacteria or had been isolated independently in this laboratory or elsewhere, with the exception of the Campo strain. The corynebacterial phase reacted with the PPLO sera, but a serum prepared against that

phase reacted only with the homologous antigen, presumably because the surface antigens were mainly represented, and were absent from the PPLO.

Preparations for examination with the optical and electron microscopes were made from the corynebacterium colonies, from the PPLO colonies, and from mixtures of the two forms when these had apparently arisen from one form only; that is to say, from plates on which both organisms occurred which had been inoculated with material derived from a pure culture of one form only. The corynebacterium was septate and varied from rods to coccal forms (Pl. 1, fig. 1). Preparations of mixtures of corynebacteria and PPLO, which had apparently arisen from the corynebacteria only showed flat, disk-like bodies, filaments and small granules (Pl. 1, fig. 2, 3).

Table 3. *Gel diffusion tests*

	Antigens											
			PPLO isolates					Edward, type 1 PPLO				Edward, type 2
Antisera	Bacterial phase (0)	PPLO (0)	1	2	3	4	5	H26	H23	H5OR	D419	Campo
PPLO (1)	+	+	+	+	+	+	+	+	+	+	+	−
PPLO (1)	±	+	+	+	+	+	+	+	+	+	+	−
PPLO (P)	+	+	+	−	−	−	−	+	−	−	−	−

After the PPLO had been subcultured regularly on chocolate agar for several months, electron micrographs showed larger, more opaque, bodies which appeared to be capable of breaking down into small particles (Pl. 1, fig. 4). Preparations of mixtures of corynebacteria and PPLO which had apparently arisen from the PPLO alone showed coccal and rod-shaped bacterial forms (Pl. 2, fig. 5, 6, 7) and smaller particles of various sizes ranging down to approximately 0·1 μ in diameter (Pl. 2, figs. 5, 7).

DISCUSSION

Bacterial L-forms were originally identified with PPLO, and the suggestion that the latter are, in fact, a type of stabilized L-form has often been made (Bisset, 1952; Dienes & Weinberger, 1951; Tulasne, 1951). Recently, evidence has accumulated to suggest that corynebacteria (Minck, 1953; Wittler *et al.* 1956; Smith, Peoples & Morton, 1957) and possibly Haemophilus (Amies & Jones, 1957) may be related to PPLO of genital tract origin.

The present evidence indicates that one PPLO isolated in association with a strain of *Corynebacterium cervicis*, and several strains isolated independently from the vagina, are serologically closely related one to another, and to authentic strains of human mycoplasma Type 1, as well as to this and other strains of *C. cervicis* and to a PPLO or L-form derived from a corynebacterium by the use of penicillin. The PPLO and associated corynebacteria, in both cases, are transformable one to the other, and electron micrographs show the processes of this transformation, as well as the morphology of the PPLO, which are identifiable as such because of their appearance, origin and cultural characters, as well as their serological relationships. They are typical of PPLO, as described by Bordet (1910), Borrel, Dujardin-Beaumetz, Jeantet & Jouan (1910), Bridré & Donatien (1923), Cuckow & Klieneberger-Nobel (1955), Dienes (1960), Freundt (1960), Klieneberger-Nobel (1954), Laidlaw &

Elford (1936), Ledingham (1933), Liebermeister (1960), Nowak (1929), Sabin (1941), Tang, Wei, McWhirter & Edgar (1935), Turner (1935), Wroblewski (1931). The combination of evidence renders their identity as PPLO unquestionable, but it is also apparent that many characteristics of PPLO are shared with L-forms, and that, in relation to the associated corynebacterium, this PPLO and those of Wittler, Smith and their collaborators, must be regarded as L-forms.

Much argument has been expended upon points of difference between L-forms and PPLO, in respect of stability, fermentation pattern, etc., but it would appear that much opposition to the concept that they are alike is based upon the supposition that the L-forms most frequently studied are in some respect typical, for which there is no logical basis. Since it appears that large numbers of PPLO are closely related it is reasonable to suppose that their parent bacteria are also closely related, and probably many PPLO are L-forms of corynebacteria. It does not appear that the differences between PPLO and the L-forms of Proteus, which is the subject of almost all such comparisons, are greater than, if as great as the differences between Proteus and Corynebacterium. The general conclusion from these observations is that the type of vaginal PPLO, sometimes described as *Mycoplasma hominis* Type 1, is the L-form of *Corynebacterium cervicis*. *M. hominis* Type 2 (Campo) appears to be distinct, but has also been claimed to be associated with a corynebacterium (Smith *et al.* 1957). The descriptions of the so-called Haemophilus of Amies & Jones (1957) and Edmunds (1960) suggest that this organism may also be a corynebacterium, and that the origin of vaginal PPLO is relatively uniform.

REFERENCES

AMIES, C. R. & JONES, S. A. (1957). A description of *Haemophilus vaginalis* and its L-forms. *Canad. J. Microbiol.* **3**, 579.

BISSET, K. A. (1952). *Bacteria*. Edinburgh and London: E. and S. Livingstone.

BORDET, J. (1910). La morphologie du microbe de la péripneumonie des bovidés. *Ann. Inst. Pasteur*, **24**, 161.

BORRELL, A., DUJARDIN-BEAUMETZ, E., JEANTET & JOUAN (1910). Le microbe de la péripneumonie. *Ann. Inst. Pasteur*, **24**, 168.

BRIDRÉ, J. & DONATIEN, A. (1923). Le microbe de l'agalaxie contagieuse et sa culture *in vitro*. *C. R. Acad. Sci., Paris*, **177**, 841.

CUCKOW, F. W. & KLIENEBERGER-NOBEL, E. (1955). Further studies of the organisms of the pleuropneumonia by electron microscope. *J. gen. Microbiol.* **13**, 149.

DIENES, L. (1960). Controversial aspects of the morphology of PPLO. *Ann. N.Y. Acad. Sci.* **79**, 356.

DIENES, L. & WEINBERGER, H. J. (1951). The L-forms of bacteria. *Bact. Rev.* **15**, 245.

EDMUNDS, P. N. (1960). *Haemophilus vaginalis*: morphology, cultural characters and viability. *J. Path. Bact.* **79**, 273.

FREUNDT, E. A. (1960). Morphology and classification of the PPLO. *Ann. N.Y. Acad. Sci.* **79**, 312.

KLIENEBERGER-NOBEL, E. (1954). The organisms of the so-called pleuropneumonia group. *Biol. Rev.* **29**, 154.

LAIDLAW, P. P. & ELFORD, W. J. (1936). A new group of filterable organisms. *Proc. Roy. Soc.* B, **120**, 292.

LAUGHTON, N. (1951). Observations on the bacterial flora of the cervical canal in infertile women with cervical dysfunction. *Proc. Soc. Study Fertil.* **3**, 18.

LAUGHTON, N. (1954). A study of a bacterium isolated from the human cervix. *J. Path. Bact.* **67**, 169.

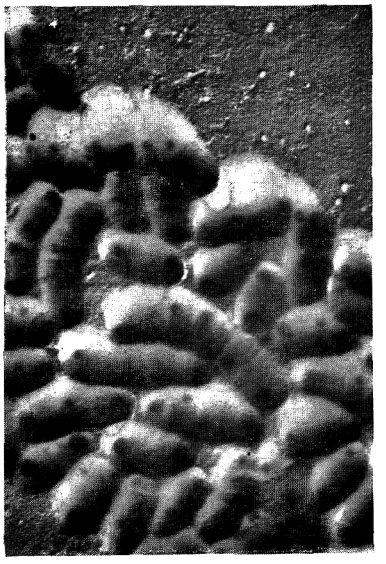


Fig. 1

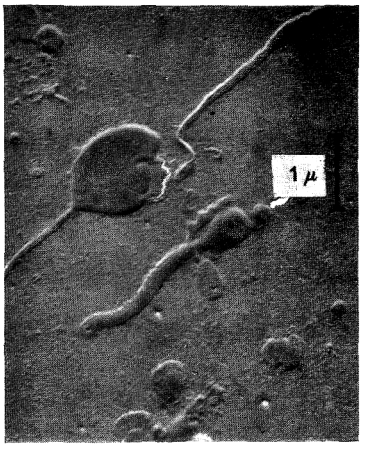


Fig. 3

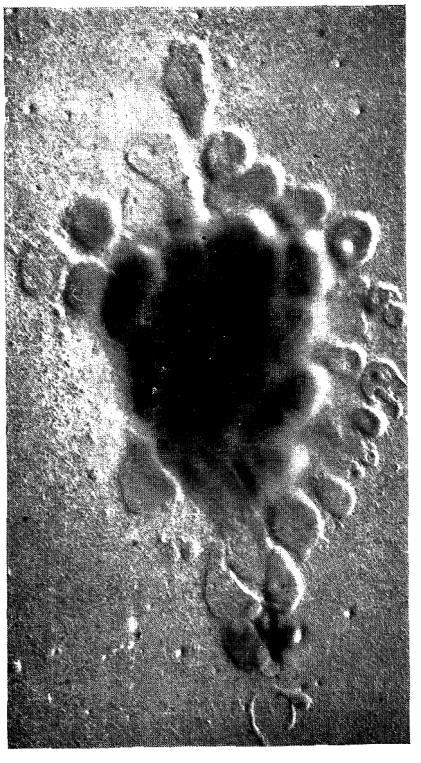


Fig. 2

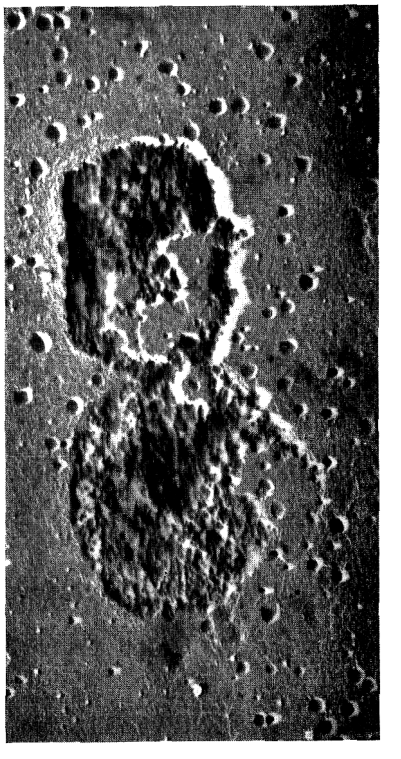


Fig. 4

(*Facing p.* 388)

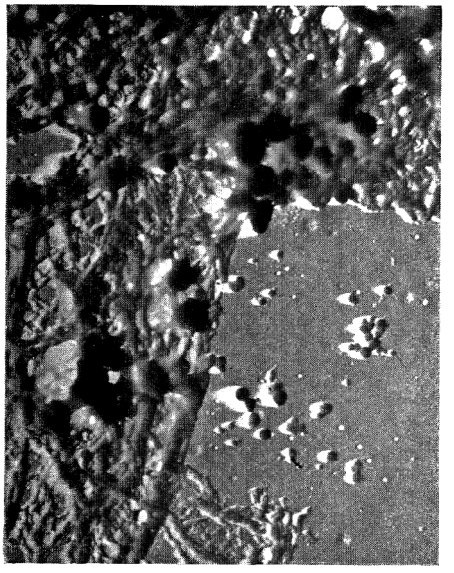


Fig. 5

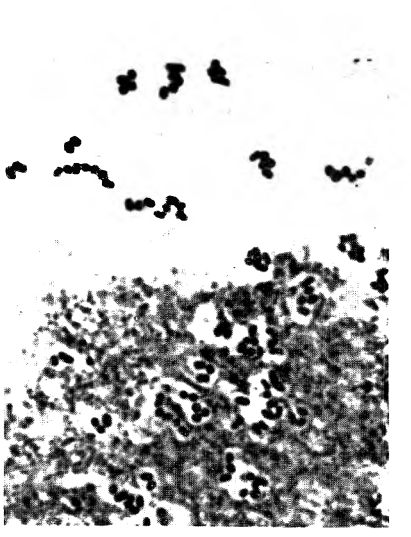


Fig. 6

Fig. 7

LEDINGHAM, J. C. G. (1933). The growth phases of pleuropneumonia and agalactia on liquid and solid media. *J. Path. Bact.* **37**, 393.

LIEBERMEISTER, K. (1960). Morphology of the PPLO and L-forms of *Proteus*. *Ann. N.Y. Acad. Sci.* **79**, 326.

MINCK, R. (1953). Recherches sur l'origine des organisms du type de la péripneumonie trouvés dans les organes génitaux de la femme. *C.R. Acad. Sci., Paris*, **236**, 250.

NOWAK, J. (1929). Morphologie, nature et cycle évolutif du microbe de la péripneumonie des bovidés. *Ann. Inst. Pasteur*, **43**, 1330.

SABIN, A. B. (1941). The filterable organisms of the pleuropneumonia group. *Bact. Rev.* **5**, 1.

SMITH, P. F., PEOPLES, D. M. & MORTON, H. E. (1957). Conversion of pleuropneumonia-like organisms to bacteria. *Proc. Soc. exp. Biol., N.Y.* **96**, 550.

TANG, F. F., WEI, H., MCWHIRTER, D. L. & EDGAR, J. (1935). An investigation of the causal agent of bovine pleuropneumonia. *J. Path. Bact.* **40**, 391.

TULASNE, R. (1951). Les formes L des bactéries. *Rev. Immunol.* **15**, 223.

TURNER, A. W. (1935). A study on the morphology and life-cycles of the organism of *Pleuropneumonia contagiosa boum* (*Borrelomyces peripneumonie* nov. gen.) by observation in the living state under dark-ground illumination. *J. Path. Bact.* **41**, 1.

WITTLER, R. G., CARY, S. G. & LINDBERG, R. B. (1956). Reversion of a pleuropneumonia-like organism to a corynebacterium during tissue culture passage. *J. gen. Microbiol.* **14**, 763.

WROBLEWSKI, W. (1931). Morphologie et cycle évolutif des microbes de la péripneumonie des bovidés et de l'agalaxie contagieuse des chévres et des moutons. *Ann. Inst. Pasteur*, **47**, 94.

EXPLANATION OF PLATES

PLATE 1. Electron micrographs × 10,000.

Fig. 1. *Corynebacterium cervicis.*

Figs. 2 and 3. Impression preparations from plate inoculated with yolk fluid from egg inoculated with the corynebacterium alone. Fig. 2 shows bacterial forms surrounded by PPLO elements. Fig. 3 shows characteristic PPLO elements. 24 hr. growth.

Fig. 4. PPLO form of *Corynebacterium cervicis* after several months regular subculture. 3 hr. growth.

PLATE 2

Fig. 5 and 7. Impression preparations from plate inoculated with yolk fluid from an egg inoculated with the PPLO form of *Corynebacterium cervicis* alone. Fig. 5 shows coccal bacterial forms and smaller particles of various sizes. Fig. 7 shows rod-shaped and coccal bacterial forms and smaller particles. Electron micrographs × 10,000.

Fig. 6. Impression preparation from the same material as that in Figs. 5 and 7, showing a larger field. Similar rod-shaped and coccal bacterial forms are shown arising within the PPLO material. Photomicrograph. × 1800.

J. gen. Microbiol. (1962), 27, 391–395
With 2 plates
Printed in Great Britain

Electron Microscopic Study of the Nuclei in a *Sorangium* species

By A. A. IMSHENETSKY and V. V. ALFEROV
Institute of Microbiology, U.S.S.R. Academy of Sciences, Moscow

(*Received* 4 *April* 1961)

SUMMARY

Electron microscopy of ultrathin sections enabled the fine structural peculiarities of a myxobacterium (*Sorangium* sp.) to be studied. The myxobacteria did not show the dense membrane characteristic of eubacterial cells. A very thin membrane in the myxobacteria cells was not distinguishable from the cytoplasm. In ultrathin sections of the myxobacteria there was observed a broad dark coloured cytoplasmic layer, a light mesh-like nuclear vacuole located in the centre of the cell and a rather large nucleus situated in the vacuole; the nucleus displayed density, dark colour and roundness. The elongated myxobacteria cells may include two to three nuclei. Phase-contrast microscopy of living unstained organisms of the *Sorangium* sp. studied revealed large rounded nuclei. The observations of living cells permitted the amitotic division of nuclei which precedes cell division in myxobacteria to be studied. The studies of the ultrathin sections with the aid of electron microscopy and the microscopy of the living cells supply similar data about the fine structure of the myxobacterial cells. The structure of the myxobacterial cells is more complicated than in the Eubacteria. The peculiar life cycle and the existence of a large nucleus easily detectable (by various methods) enables one to place the myxobacteria among the relatively highly organized micro-organisms.

INTRODUCTION

Fine detail in the structure of true bacteria (Eubacteriales) continues to be a subject of lively argument (DeLamater, 1960; Bissett, 1952). On the other hand, the study of the structure of the myxobacterial cell usually led to rather close agreement. Seventy years have elapsed since the appearance of Thaxter's (1892) classical work which described the Myxobacteriales for the first time. All those who have been engaged in the study of cell structure in myxobacteria since then have continually observed rather large and easily distinguishable nuclei, differentiated morphologically from cytoplasm and demonstrated without any difficulty by the common cytological methods.

Beebe (1941), Klieneberger-Nobel (1947), Loebeck & Ordal (1957) studied the cytology of myxococci in detail and Grace (1951) that of the Sporocytophaga. In spite of some disagreement in interpretation of microscopic pictures, nuclei were distinguished in all cases with the myxobacteria. Under the electron microscope it was shown that nuclei of myxococci may appear dark or light, though it is remarkable that in fixed cells it was impossible to detect the dark nuclei (Loebeck & Ordal, 1957). However, in the course of studying the biology of myxobacteria it became evident that bacteria not forming fruiting bodies and exhibiting a reduced life cycle

belonged to the same group and these were named Promyxobacteria (Imshenetsky & Solntzeva, 1945). The peculiar feature of these more primitive forms is that some species contain deoxyribonucleic acid, but are devoid of a morphologically discrete nucleus. The representatives of the genus *Cytophaga*, which include species which decompose cellulose and species devoid of this capacity (Stanier, 1942; Imshenetsky & Solntzeva, 1936) may be grouped with these primitive forms, where a more primitive life cycle corresponds to a simpler cell organization, i.e. one lacking a nucleus. However, the majority of Myxobacteriales consisting of the genera *Myxococcus*, *Melittangium*, *Sorangium*, *Polyangium*, display large discrete nuclei. By using electron microscope techniques it has been shown for the first time that the nuclei appear denser and darker than the cytoplasm structure (Imshenetsky, 1949). In the present paper the authors give the results of electron microscope studies of ultrathin sections of a species of the genus *Sorangium*.

METHODS

The investigation was made on a pure culture of *Sorangium* sp. isolated from the cellulose mass to be used for paper production. This species was not identified further. When inoculated into Hutchinson's mineral medium (Hutchinson & Clayton, 1919) it did not decompose filter paper. On the surface of potato agar it formed a round, flat, bright, greyish white colony with a creeping margin (Pl. 1, fig. 1). The formation of fruiting bodies of macrocysts within the colony was not observed. The rod-like vegetative cells in the older cultures were reduced and converted into oval microcysts, a characteristic feature of the genus *Sorangium*.

To obtain the necessary amount of myxobacteria the following method was used. A thick watery suspension of myxobacteria taken from a culture which had grown on a slope of potato agar for 18 hr. was spread over potato-agar plates. These plates were kept in the incubator at 28° for 3 hr. The newly grown organisms were washed off with a veronal acetate buffer solution (pH 7·1). This suspension was centrifuged and a 1·5 % (w/v) solution of $KMnO_4$ in distilled water added to the deposited organisms which were then shaken thoroughly and kept for 5 min. at room temperature. After fixation the organisms were washed for 10 min. with tap water and submerged for 2 hr. in a 3 % (v/v) solution of neutral formalin (40 %, w/v, formaldehyde). Then the organisms were washed twice with ethanol (25 %, v/v) in water each time for 30 min. This fixation method provided better results than fixation with osmic acid solution (0·2 %, w/v) or $K_2Cr_2O_7$ (1 %, w/v). After fixation, two drops of melted 2 % (w/v) starvation agar in distilled water were introduced into the bottom of the centrifuge tube containing the deposit of fixed and ethanol-washed organisms. The agar was mixed with the deposit and 1 or 2 drops of this mixture transferred to a sterile glass slide. After cooling, the agar was cut with a scalpel into pieces about 1–2 mm.^{3} each. The pieces were successively treated with 50 %, 70 %, 96 % (v/v) ethanol in water for 15 min. (single changes) and then twice in absolute ethanol for 30 min. Then the agar blocks containing the myxobacteria were placed for 15 min. in a mixture of equal volumes of absolute ethanol and butyl-methacrylate, followed by three 15 min. changes in a mixture of 9 vol. butyl-methacrylate and 1 vol. methyl-methacrylate. The agar blocks were then placed in gelatin capsules and embedded in a mixture composed of butyl-methacrylate,

methyl-methacrylate and benzoin (9 ml. + 1 ml. + 0·05 g.). The capsules were closed tightly with lids. Polymerization took 8 hr. under ultraviolet radiation. Sections were cut with an ultramicrotome which enabled sections 200–300 Å thin to be obtained; these were studied with the aid of a Siemens electron microscope at an acceleration voltage of 45 kV.

RESULTS

The structure of the myxobacterial cell

It is known that some myxobacteria perform flexible movements which the true bacteria are unable to do. Thus it was of interest to investigate the specific structure of the cell membrane. As expected the cells of the myxobacteria did not show a dense well defined three-layer membrane as seen in representative Eubacteria. Even a magnification of × 50,000 did not reveal a darker layer in the peripheral layers of the cytoplasm in myxobacterial cells. This does not mean that the myxobacterial cells are devoid of a thin membrane; the latter may be revealed when the cells of the large myxobacteria are placed in a saturated solution of sodium chloride (Solntzeva, 1941). However, the electron microscope technique did not show this membrane in microphotographs of the Sorangium investigated.

When the section ran parallel with the cell axis, the cell had a cylindrical form with somewhat sharpened ends (Pl. 1, fig. 2). Three zones could be distinguished within the inner part of the cell: (*a*) The peripheral zone, i.e. the darker zone, corresponding to the rather dense layer of the cytoplasm (Pl. 1, figs. 2, 3, 5). (*b*) A lighter site displaying a mesh structure in the central part of the cell. This part of the cell seemed to be empty; in it only single net-like structures or fibres set against an entirely light background were visible. This central zone will henceforth be referred to as the nuclear vacuole. (*c*) As a rule the central part of the nuclear vacuole showed a distinct dense, rounded and well defined nucleus; in the photographs it is almost black (Pl. 1, figs. 2, 3, 5; Pl. 2, fig. 7). In rare cases the nucleus was revealed, not in the central part of the nuclear vacuole, but at its margin (Pl. 1, fig. 4). The size of the nucleus in the ultrathin slice was somewhat smaller, owing to shrinkage during fixation. It should be noted that the apparent size of the nucleus depends upon the plane of sectioning.

The plane of sectioning could affect the cell contour. For example, a lateral sectioning of the cell could be responsible for the cell's apparent spindle-shaped or sphenoid form. Its terminal points could be sharpened (Pl. 1, figs. 3, 4). When the microtome knife cut the cell through the nucleus, the cell's contour displayed a proper rounded form and all the three mentioned zones were observed quite distinctly: the dark broad cytoplasmic ring which exhibited minute grain structure, a slightly oval-shaped light nuclear vacuole and a dark round nucleus (Pl. 2, fig. 6). In small cells, as a rule, there was only one nucleus. However, the division of the nucleus may occur much earlier than cell fission; the typical pattern of the latter in myxobacteria was transverse binary fission. In this case the elongated cell showed 2–3 nuclei located at a considerable distance from each other (Pl. 2, figs. 7, 8). Sometimes, the section ran through the cell above or below the nucleus and then the photographs showed cells containing only cytoplasm and the nuclear vacuole, but devoid of the dark round nucleus (see Pl. 1, fig. 3; Pl. 2, figs. 7, 8).

The comparative study of the electron micrographs with microphotographs of

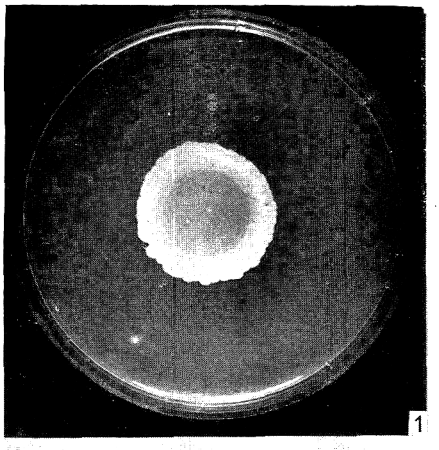
living unstained Sorangium cells is of interest. Phase-contrast microscopy in living cells revealed the nuclei most distinctly (Pl. 2, figs. 9, 10). In living myxobacteria the nuclei appeared larger than the cell nuclei observed in the ultrathin sections. This may be attributed to shrinkage of the nuclei under conditions of 'severe' fixation and the treatment which the cells received before the ultrathin sectioning.

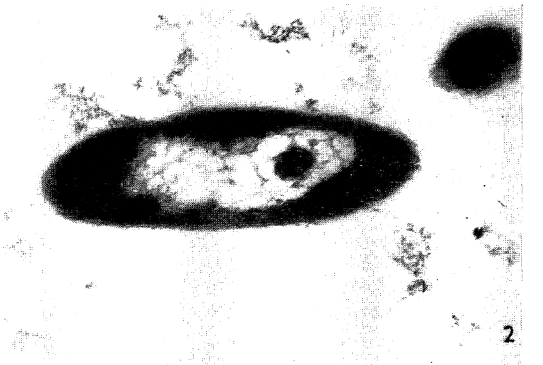
The observation of living myxobacteria enabled the cell division pattern by segmentation which took place before cell division by fission to be traced. Similar dividing cells are marked with arrows in Pl. 2, figs. 9 and 10. In living cells as well as in the ultrathin cell sections two nuclei were sometimes seen. Similar cells are represented in Pl. 2, fig. 9, and are marked as 'D'. The microscopy of living young Sorangium cells revealed a lighter, rather wide zone surrounding the nucleus; this zone may possibly correspond to the nuclear vacuole observed in the ultrathin sections. These light passages were especially well seen at the end of the lag phase, i.e. 3–4 hr. after the re-inoculation of the culture on to fresh nutrient medium. The results of studying the structure in living Sorangium cells will be presented in a separate paper.

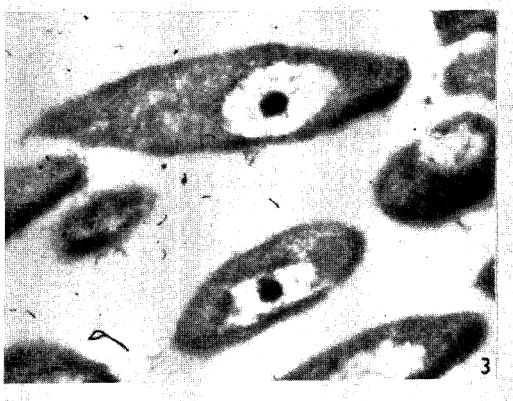
DISCUSSION

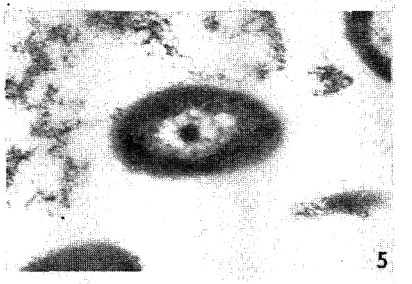
Both electron microscopy of ultrathin sections and phase-contrast microscopy of living cells gave identical results for the investigation of nuclei in myxobacteria. The form, localization and behaviour of the nuclei turned out to be of analogous character. From the viewpoint of comparative cytology it should be noted that electron microscope techniques revealed that in myxobacteria the nuclei appeared to be much denser than the cytoplasm and, similar to the picture shown in plant and animal cells, they were much darker than the cytoplasm. The high content of deoxyribonucleic acid in the myxobacteria (Belozersky, 1959) is in agreement with the presence of rather large nuclei. The physico-chemical features of this nuclear structure would explain the retardation of the electron beam and hence the appearance of dense dark nuclear structures in the microphotographs of ultrathin sections. It should be emphasized that electron microscopy of true bacteria (Eubacteriales) does not reveal similar dark structures and the nuclear vacuoles are often described as the nucleus, shown as large light sites located in the central part of the cell. The division of nuclei in myxobacteria follows an amitotic pattern and usually precedes the cell division process.

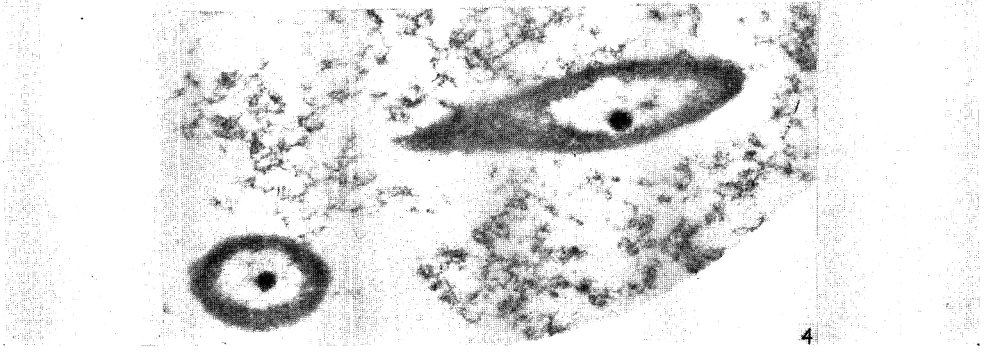
The myxobacterial nuclei observed in unstained living organisms shows that phylogenetically this group is much higher than the true bacteria (Eubacteriales). Study of the detailed karyologic characteristics of the myxobacteria is a task for future investigations, but even at present it is evident that the complex life cycle of the myxobacteria, the formation of fruiting bodies in particular, harmonizes with the complicated organization of the organism itself. Therefore it is hard to agree with the suggestion that the myxobacteria and the eubacteria, particularly the sporogenous bacilli, have an identical nuclear apparatus (Badian, 1933). The polyphyletic origin of bacteria is indisputable and amongst them it is possible to distinguish both more and less complicated organisms.

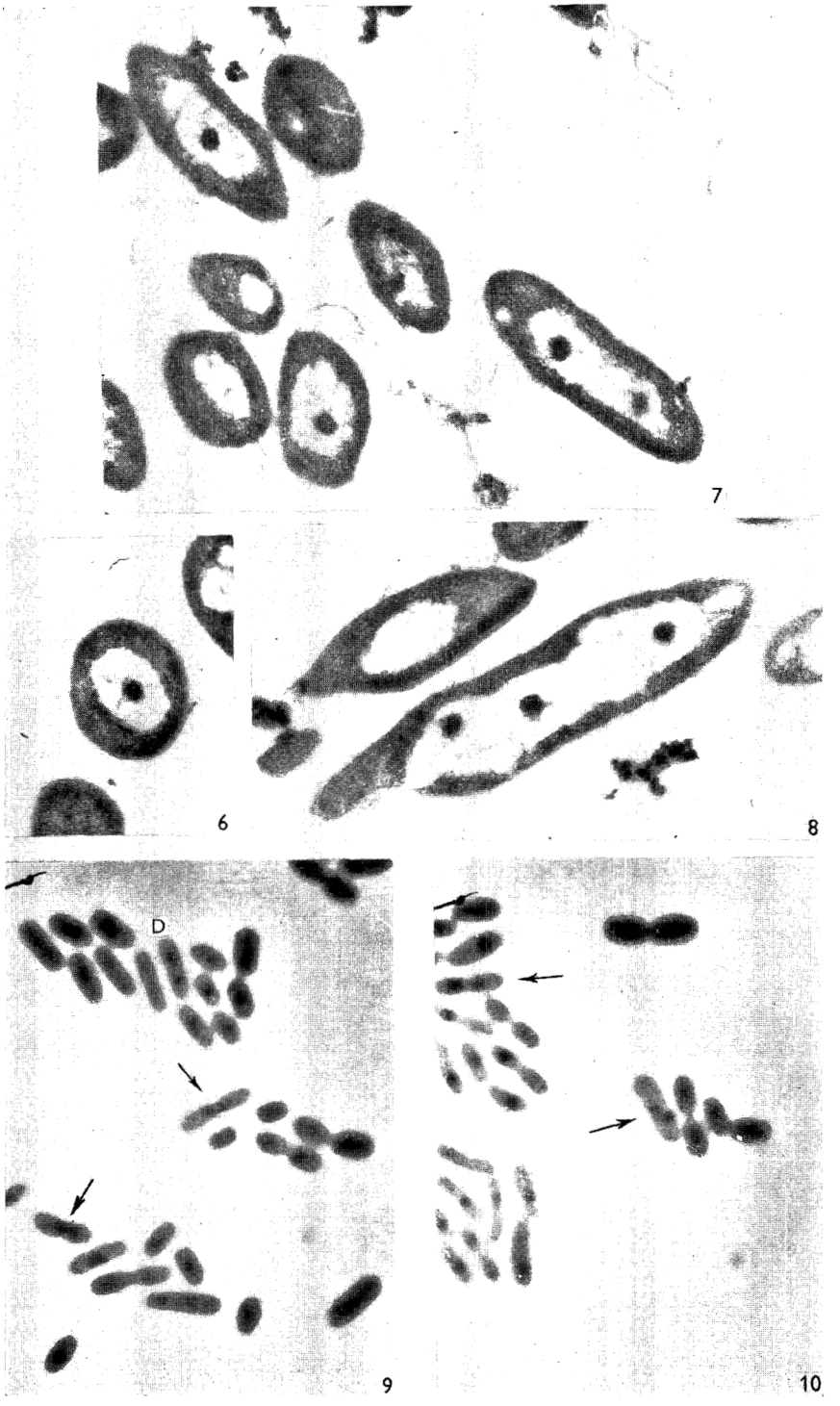












A. A. IMSHENETSKY AND V. V. ALFEROV

REFERENCES

BADIAN, J. (1933). Eine cytologische Untersuchung über das Chromatin und den Entwicklungszyklus der Bakterien. *Arch. Mikrobiol.* **4**, 409.

BEEBE, J. (1941). The morphology and cytology of *Myxococcus xanthus*, n.sp. *J. Bact.* **42**, 193.

BELOZERSKY, A. (1959). *Nucleoproteidi i nucleinovie kisloti rastenij ikh biologicheskoje znachenije.* Moskva.

BISSET, K. (1952). The interpretation of appearances in cytological staining of bacteria. *Exp. Cell Res.* **3**, 681.

DELAMATER, E. (1960). *Chromosomi bacterij i mekhanism ikh delenija. Anatomija bacterij.* Moskva.

GRACE, J. (1951). The life cycle of Sporocytophaga. *J. gen. Microbiol.* **5**, 519.

HUTCHINSON, H. & CLAYTON, I. (1919). On the decomposition on cellulose by an aerobic organism (*Spirochaeta cytophaga*, n.sp.). *J. agric. Sci.* **9**, 141.

IMSHENETSKY, A. (1949). Electronnomicroscopichescoye isuchenye micsobacteriy. *Microbiology, Moscow*, **18**, 393.

IMSHENETSKY, A. & SOLNTZEWA, L. (1936). Ob aerobnikh cellulosnikh bacteriyakh. *Bull. Acad. Sci. U.R.S.S. (Sér. Biol.)*, **6**, 1115.

IMSHENETSKY, A. & SOLNTZEWA, L. (1945). O nesowershenikh formakh micsobacteriy. *Microbiology, Moscow*, **14**, 22.

KLIENEBERGER-NOBEL E. (1947). A cytological study of Myxococci. *J. gen. Microbiol.* **1**, 33.

LOEBECK, M. & ORDAL E. (1957). The nuclear cycle of *Myxococcus fulvus*. *J. gen. Microbiol.* **16**, 76.

SOLNTZEWA, L. (1941). Biologiya micsobacteriy. II. Rod Melittangium i rod Chondromyces. *Microbiology, Moscow*, **5**, 505.

STANIER, R. (1942). The Cytophaga group: a contribution to the biology of myxobacteria. *Bact. Rev.* **6**, 143.

THAXTER, R. (1892). On the Myxobacteriaceae, a new order of Schizomycetes. *Bot. Gaz.* **17**, 389.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. A giant colony of *Sorangium* sp. in the Petri dish of potato agar.

Fig. 2. An ultrathin section of the *Sorangium* sp. cell cut in parallel with the cell's long axis. The cytoplasm, light nuclear vacuole and the dark nucleus are visible. The cell's thin membrane is not discernible (× 50,000).

Fig. 3. The oblique ultrathin section reveals a spindle-like cell contour. The sections reveal the nuclei, a nuclear vacuole, and dark cytoplasm (× 40,000).

Fig. 4. As a result of oblique sectioning the cell displays a sharpened end (× 40,000).

Fig. 5. The sectioning of a small-sized juvenile cell. A dark cytoplasm and nucleus. Light nuclear vacuole (× 40,000).

PLATE 2

Fig. 6. A transverse section of the rod-like *Sorangium* sp. cell. A light nuclear vacuole with a nucleus in the centre. The vacuole is surrounded with a dark cytoplasmic ring (× 40,000).

Fig. 7. Two nuclei in the elongated *Sorangium* sp. cell. In some cells the nuclei are not seen because the section was either above or below the nucleus (× 40,000).

Fig. 8. Three nuclei are located in the light nuclear vacuole of the elongated Myxobacteria cell. In the second cell the section is either above or below the nucleus, which is therefore not seen in the cell (× 40,000).

Fig. 9. The living non-stained *Sorangium* sp. cells. The arrow marks cells containing the dividing nucleus. Letter 'D' denotes cells having two nuclei. Phase contrast (× 4500).

Fig. 10. Nuclei in the living Myxobacteria cells. Cells with dividing or recently divided nuclei are marked with arrows. Phase contrast (× 4500).

J. gen. Microbiol. (1962), **27**, 397–414
Printed in Great Britain

Purification of Rabies Soluble Antigens

By T. H. MEAD

The C.S.I.R. and U.C.T. Virus Research Unit, Department of Pathology, University of Cape Town, South Africa

(*Received* 28 *April* 1961)

SUMMARY

The ability of extracts of rabies-infected suckling mouse brains, freed from virus by centrifugation, to fix complement in the presence of mouse anti-rabies serum was confirmed. The complement-fixing (CF) activity, which was unstable, was partially purified by precipitation, solvent treatment, adsorption, chromatography and electrophoresis. There appeared to be constituents of normal brain which closely resembled the CF activity in their behaviour towards these treatments. Descriptions are given: of (*a*) a device for stepwise elution of chromatograph columns; (*b*) modifications to the LKB Produkter electrophoresis apparatus to ensure constant electrical resistance; (*c*) a method for estimating 'protein' which is useful when the ultra-violet (u.v.) radiation method is inapplicable.

INTRODUCTION

It was shown by Polson & Wessels (1953) that extracts of rabies-infected suckling mouse brains contained in addition to the virus (which could be removed by centrifugation) a smaller specific component which was demonstrable by complement-fixation tests. Much additional information about the antigen was published by Van den Ende, Polson & Turner (1957). The original objects of the work now presented were to purify this small component, to compare it with the intact virus and to attempt to determine whether it was a subunit of the virus particle or merely a by-product of infection.

METHODS

Virus. The history of the egg-adapted Flury strain of virus was described by Van den Ende *et al.* (1957). Material from the seventeenth passage in suckling mouse brain was freeze-dried and used as reference stock. From this, working stocks were prepared at intervals and kept at $-70°$. The total number of passages did not exceed 20. In May 1960 it was confirmed that the current working stock virus (LD50 = $10^{-5\cdot0}$/0·02 ml. intracerebrally in mice) was completely neutralized (LD50 < 10) by a rabbit anti-rabies serum prepared in Johannesburg by the South African Institute for Medical Research.

Mouse anti-rabies sera. Adult mice in batches of about 200 received 10–13 0·2 ml. intraperitoneal injections of a 10 % (v/v) emulsion of freshly infected suckling mouse brains at 3- to 4-day intervals. Usually, in the preparation of batches VII and IX, for example, only the first dose was inactivated with formalin (0·35 %). Serum VIII which later proved to differ perceptibly from other batches was pre-

pared by immunization with formalin-inactivated virus throughout. Mice were bled 7–10 days after the last injection. The serum was inactivated at 56° for at least 30 min., clarified by centrifugation at 59,000 *g* for 1 hr. under liquid paraffin (which assisted the removal of lipids) and stored at 4° after treatment with thiomersalate.

Normal mouse antiserum was similarly prepared by inoculating a suspension of uninfected suckling mouse brains into adult mice.

Trypsin which had been crystallized at least twice was the generous gift of Seravac Laboratories, Cape Town. Some batches were salt free, others contained about 30 % of magnesium sulphate.

Complement-fixing titrations were carried out generally as described by Casals & Olitsky (1950) but non-serial logarithmic dilutions covering a range of 1 log in 10 steps were used instead of serial twofold dilutions for all but the less exact experiments. The smoothness of the curves obtained by plotting the CF titres of successive fractions from chromatography and electrophoresis appeared to justify the assumption that differences of 0·2 log (1·58 fold) obtained in one test were probably significant. When results obtained on one day were to be compared with those found on another, a standard batch of freeze-dried extract was titrated on both occasions and the results were corrected for any discrepancy between the titres of the standard. The diluent for the antigen (CFT saline) was the veronal-buffered saline containing Mg and Ca recommended by Mayer, Osler, Bier & Heidelberger (1946).

Chemicals. Reagents were of the best quality available and solvents were redistilled through a column. All water was distilled in glass. 1:2-Difluorotetrachloroethane (Freon 112) was a gift from E. I. du Pont de Nemours and Co. Difco agar was treated by the method of Dulbecco & Vogt (1954).

Buffered saline (BS), 0·85 % (w/v) NaCl, 0·01 M-phosphate (pH 7); pH 8·4 *saline*, 0·85 % (w/v) NaCl, 0·02 M-Na_2HPO_4 adjusted to pH 8·4 with 0·2 M-KH_2PO_4.

EDTA saline, NaCl 17 g., disodium ethylenediaminetetra-acetate (EDTA) 7·4 g., 0·2 M-Na_2HPO_4 63 ml., 'Triton X-100' (Rohm and Haas Co.) 0·2 g. thiomersalate 0·2 g., 2N-NaOH to pH 7·0 in 2 l. All other buffers contained 'Triton X-100' 0·01 % (w/v) and thiomersalate 0·01 % (w/v).

Centrifugation at values exceeding 4000 *g* was carried out in models L or LH Spinco centrifuge. The forces quoted are average values calculated at the central point of the liquid column. For lower forces an MSE refrigerated centrifuge was used.

Methods of concentration. Pervaporation with intermittent dialysis against thiomersalate water followed by freeze-drying in ampoules was usually used. Prolonged dialysis (for more than 24–36 hr.) caused precipitation and was avoided. Pervaporation after being started in a current of warm air was sometimes continued overnight by placing the cellulose sac on a Petri dish under the cooling element of the refrigerator. In the morning the ice surrounding the sac and the small frozen residue inside were allowed to melt undisturbed to avoid cutting the cellulose. In some experiments dialysis against polyethylene glycol (Kohn, 1959) was used, but occasionally the low molecular weight (6000) glycol locally available passed through the membrane. The tendency of parts of the solution to dry on the sac and the difficulty in bringing the residue to a very small known volume (usually 0·05 ml. for Ouchterlony tests) were disadvantages of this method, for our purposes.

Comminution of brains was done at about 14,000 rev./min. in a 'top drive' blender

in which all parts including the ball bearings were of stainless steel, enabling autoclaving to be carried out without the formation of rust.

Total nitrogen was determined by micro-Kjeldahl with the mercury catalyst and digestion conditions of McKenzie & Wallace (1954).

Estimation of 'protein'. In some early experiments total dry residue (after dialysis) or total nitrogen were used as guides to the concentration of substances (mostly impurity) in experimental solutions. As the number of samples increased and their concentration diminished, alternative methods were tested. Because of uncertainties in u.v. absorption measurements and insensitivity of other methods, 'protein' was determined by measuring the light scattered by the precipitate formed in very dilute protein solutions by the addition of perchloric acid. All observations made by the method will therefore be referred to as estimates of 'protein' with exclusion of such non-precipitable mucoproteins as may have been present. The method was: to 1 ml. of solution diluted to contain about 0·05–0·2 mg. protein/ml. was added 3 ml. of dilute (about 8 %, w/v) perchloric acid (43·3 ml. A.R. 60%, w/w, acid diluted to 500 ml.). A blank was set up at the same time and also a standard consisting of 1 ml. of a 1/1000 dilution of human serum in thiomersalate saline. This standard was kept at 4° and used throughout and on the assumption that it contained about 0·07 mg./ml. of protein rough correlation with actual protein content could be made. Light scattering reached a maximum in 10 min. and remained constant for at least 30 min. Measurements were made in a dark room with an apparatus based on that of Oster (1950). Unfiltered light from a 125 W mercury lamp, rendered parallel by a condenser and collimated by two vertical rectangular slits, entered one side of a rectangular Perspex cell internally 1 cm. × 1 cm. The photomultiplier cell used as detector was supported at right angles to the incident beam in a housing having two vertical slits 8 cm. apart, excluding all but a narrow beam entering from the centre of the Perspex cell. Two adjacent sides of the cell were clear and two black. A square depression in the cell support ensured accurate placement. The output from the photocell was measured by a Cambridge 'spot' galvanometer (475 Ω, deflexion 125 scale divisions/μA.) with a potentiometer for varying the sensitivity. The batteries and galvanometer rested on 'Perspex' insulators and an extension handle of the same material was fitted to the zero adjuster. In use, the galvanometer was set to zero with the blank cell in position and (using the sensitivity control) to 19·2 divisions with the standard and these two mutually dependent adjustments were repeated as required. The cell containing the experimental solution was then inserted and the galvanometer reading recorded. In any series of observations the zero point with the blank was checked between every measurement. The lamp was supplied from a constant-voltage transformer but mains voltage fluctuations were sometimes perceptible in spite of this precaution. 'Protein' estimates quoted in this paper are the galvanometer readings obtained when 1 ml. of the given dilution was treated with 3 ml. of the perchloric acid solution. Tests made with dilutions of crude and purified mouse brain extracts showed that the relation between concentration and deflexion was linear between 2 and 100 on the galvanometer scale.

Chromatography. Calcium phosphate suspension (brushite and hydroxyapatite) were prepared as described by Tiselius (1954) and Tiselius, Hjertén & Levin (1956). Diethylaminoethylcellulose (DEAE cellulose) and carboxymethylcellulose (CM

cellulose) were prepared according to Peterson & Sober (1956). Triethylaminoethylcellulose (TEAE cellulose) was prepared from DEAE cellulose by the method of Porath (1957).

Stepwise elution, when continued overnight, was by buffers layered over one another

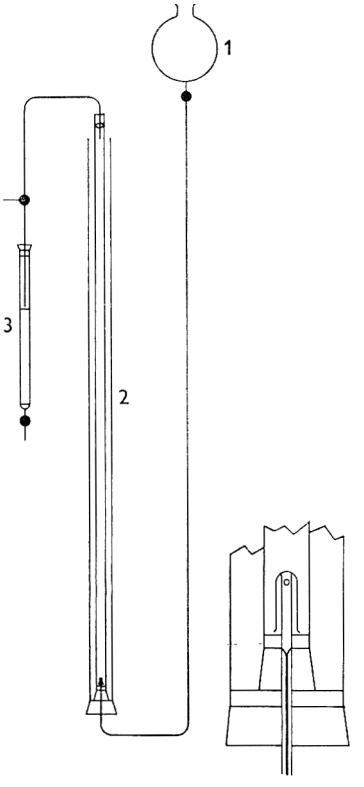


Fig. 1

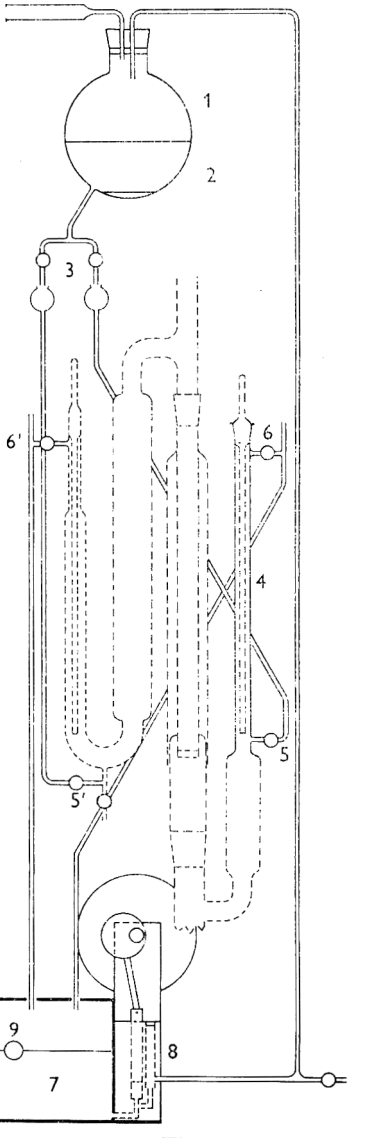


Fig. 2

Fig. 1. Apparatus for stepwise elution. 1, separating funnel; 2, reservoir; 3, column. Connexions are of narrow bore tube. Inset shows, on a larger scale, the baffle preventing the formation of eddies in the reservoir.

Fig. 2. Buffer circulation system for the L.K.B.-Produkter column electrophoresis apparatus. Broken lines show parts of the original apparatus. Full lines show the modifications. 1, upper reservoir; 2, chloroform to maintain sterility; 3, drip feeds; 4, narrowed anode vessel; 5, 5′, buffer inlets to electrode vessels; 6, 6′, buffer exits with antisiphoning tees; 7, lower reservoir; 8, 'Perspex' pump; 9, float controlling pump.

in a tall, narrow, vertical tube surrounded by a jacket of static water to hinder convection and connected as shown in Fig. 1. The buffers were introduced through the separating funnel, starting with the most dilute, while air escaped through the tap. The funnel was eventually filled with enough of a solution denser than the most concentrated buffer, to displace the contents of the reservoir. In spite of inevitable diffusion at the interfaces, the elution diagrams had the pronounced peaks characteristic of stepwise elution. Linear gradients used in all the later experiments were prepared in apparatus based on the principle of Parr (1954). The fraction collectors were of the siphon balance type.

Electrophoresis. (*a*) *In starch gel.* The Smithies (1955) technique was used. The walls of the trough were in two sections assembled sandwichwise to facilitate horizontal slicing of the gel before staining. (*b*) *On paper and glass fibre sheets.* The apparatus of Flynn & de Mayo (1951) was used. (*c*) *On cellulose acetate strips.* The strips (Kohn, 1957) were supported on cooled glass (made water-repellent with dimethyldichlorosilane) in a closed apparatus. (*d*) *Immunoelectrophoresis and electrophoresis in agar.* The macro- and micro-methods of Grabar & Williams (1955) and of Scheidegger (1955) were used. In some experiments the thin glass plates supporting the agar slabs were placed in the closed apparatus used for starch grain electrophoresis (below) and cooled by the circulation of refrigerated water. Thermal contact between the glass and the Perspex floor of the apparatus was improved by a thin layer of 0·5 % aqueous agar. In this way gradients up to 16 V./cm. in the gel could be used without appreciable evaporation. Agar electrophoretograms were usually fixed with ethanolic acetic acid containing $HgCl_2$ (0·4 %, w/v) and stained with naphthalene black (Uriel & Grabar, 1956). Immunoelectrophoretograms after being washed with thiomersalate saline for at least 3 days were usually subjected to contact printing both before and after being stained (without prior dying) with nigrosine (0·002 %, w/v) in 2 % (v/v) acetic acid (Kohn 1958). (*e*) *Starch grains.* The technique and water-cooled apparatus of Paigen (1956) were used. The dimensions of the slab were 30 cm. × 8 cm. × about 0·5 cm. (*f*) *In a column of modified cellulose.* The column filling was prepared as described by Flodin & Kupke (1956). In using the LKB-Produkter column electrophoresis apparatus it was found that the resistance increased considerably during extended runs and it was suspected that much of this increase occurred at the electrodes and was accompanied by a decreased potential gradient in the separation zone. The apparatus was therefore modified (Fig. 2) to permit a continuous flow of recirculated buffer over the electrodes. The resistance of the modified apparatus remained constant for at least 24 hr.

Gel precipitin tests. Scarcity of antigen and antiserum restricted most testing to the micro-Ouchterlony method. After experiments with various buffers, 0·85 % (w/v) saline was used as solvent for the agar (1 %, w/v). For most tests a cutter was used to give a hexagonal arrangement of 2 mm. diameter wells with centres 5 mm. apart ('narrow spacing'). Occasionally patterns of similar holes with centres 7·5 or 10 mm. apart were used ('wide spacing'). The plates were sometimes used for contact printing before they were washed and stained. Washing in thiomersalate saline was continued for at least 3 days but frequently failed to remove all residual unprecipitated protein. The plates were usually stained in the swollen state with 0·002 % (w/v) nigrosine in 2 % (w/v) acetic acid. In some experiments duplicate plates were washed, dried and stained with naphthalene black (Uriel & Grabar,

1956) but this more complicated technique did not offer any special advantage. The main difficulties were the complete removal of irrelevant protein before staining and the photographic reproduction of the lines, often very faint.

Extraction of antigens. The brains from suckling mice dead or moribund 3–4 days after intracerebral infection with virus, were stored, when necessary, for short periods at −20° and extracted in batches of about 200 by one of the methods described below. The Casals (1949) method, modified to include extraction of the macerated brains with chloroform + methanol as well as acetone + ether, was used for most of the early experiments. Plain saline extraction was used later. When an ultrasonic disintegrator became available saline extraction by using this instrument was used. The following experiment (Table 1) made to compare the three methods, illustrates the procedures. The frozen brains from 529 infected

Table 1. *Properties of crude extracts obtained by three methods from one batch of brains*

Batch	Preparation	Volume (ml.)	CFT*	'Protein' (undiluted)†	Purity CFT/ 'protein'	Yield vol. × CFT
65/1	Saline extract	300	285	450	0·63	85,000
65/2	Saline with ultrasonic disintegration	300	500	425	1·18	150,000
65/3	Modified Casals	100	690	1000	0·69	69,000

* CFT = complement-fixing titre.

† 'Protein' undiluted = galvanometer reading × dilution factor (see under 'Methods').

suckling mice were divided into three equal portions of 33·5 g. One portion (batch 65/1) was macerated (for a total time of 2 min. at about 14,000 rev./min.) with a mixture of thiomersalate treated and cooled BS (225 ml.) and crushed ice (from distilled water, 75 g.). The mixture was centrifuged in the No. 30 rotor for 2 hr. at 44,000 *g* with refrigeration. The clear supernatant fluid formed the crude extract. Another portion (65/2) of the same batch of brains was macerated with BS and ice in the same way but before being centrifuged the mixture was submitted, in portions of 25 ml., to the maximum output at 20 Kcyc./sec. from an MSE-Mullard ultrasonic generator with a probe uniformly $\frac{3}{4}$ in. in diameter. Each portion was treated for 5 min. The main bulk of the mixture and the portion under treatment were surrounded by ice. The crude extract was obtained by centrifugation at 44,000 *g* for 2 hr. The third portion (65/3) was macerated for 2 min. in acetone (400 ml.) which had been precooled in the apparatus to −10°. The mixture was centrifuged with refrigeration at 580 g. The supernatant fluid was discarded and the sediment extracted successively with acetone (800 ml.) acetone + ether (1:1, 400 ml.) then twice with chloroform + methanol (3:1, 300 ml. each time) and finally twice with ether using 400 ml. for the penultimate and 200 ml. for the final treatment. Each extraction was carried out near 4° by intermittent shaking followed by centrifugation. After removal of ether under diminished pressure, the powder (2·78 g.) was stirred at about 4° with BS (40 ml.) containing Triton X-100 (0·01 %, w/v) and thiomersalate (0·01 %, w/v) for 30 min. The mixture was centrifuged, the supernatant fluid removed and the residue extracted twice more in the same way. The

supernatant fluids were combined and, after being centrifuged at 59,000 *g* in the No. 40 rotor for 1 hr., formed the crude extract. The Casals procedure did not seem specially advantageous but sonic disintegration appeared to increase the yield of antigen without introducing extra protein. The purities (CF titre/'protein' reading × dilution factor) of different batches (more than 60 have been used) varied from about 0·14 to 0·6. The value 1·18 for 65/2 was exceptionally high. The extracts from this particular experiment were at once purified by the acid precipitation process to be described. When not required immediately, however, crude extracts were dialysed against EDTA saline, pervaporated, dialysed against water and freeze-dried in ampoules. All extracts received, during the course of purification, two centrifugations (at 44,000 *g* in No. 30 rotor for 2 hr. or 59,000 *g* in No. 40 rotor for 1 hr.), either of which had been found by infectivity testing to remove at least 99·99 % of virus infectivity.

Attempts at stabilization. CF activity was unstable at 4° and during concentration and purification procedures. There was no loss of activity in samples stored at 4° after freeze-drying; and reduced loss after acid precipitation. Attempts were made to stabilize activity by addition of Ca and Mg ions. Extracts were also dialysed against buffer containing EDTA to remove traces of heavy metals. No convincing evidence was obtained for the stabilizing effect of any of these procedures but, owing to a suggestion of improvement, dialysis against EDTA buffer was retained as a normal step in the extraction procedure. 'Triton X-100' (0·01 %, w/v) was added to nearly all buffers and solutions because it eliminated the surface denaturation easily visible when a foam was allowed to appear on the extracts and also because its wetting action improved the performance of fraction collectors. Tests made to correlate foam-derived surface denaturation with loss of CF activity gave negative results.

RESULTS

When the presence of more than one antigen was not suspected, complement-fixing (CF) activity alone was used as a measure of antigen content and attempts were made to concentrate and purify the extracts in terms of CF titre. Attempts to separate the individual antigens are described separately (Mead, 1962).

Dialysis precipitation

A precipitate which formed during prolonged (at least 36 hr.) dialysis of crude extracts against water at 4° (or more rapidly during electrodialysis) carried down most of the CF activity. Of this about 30 % was recoverable at two to three times the original purity by extracting the precipitate with BS. Dialysis precipitation was used as a preliminary purification step in several early experiments but was superseded by acid precipitation. A precipitate which carried down part of the CF activity also formed in crude extracts kept at 37° but attempts to use this for purification were not successful.

Acid precipitation

This method was applied to extracts of infected egg membranes by Schäfer & Munk (1952) and in a different manner to mouse brain extracts by Van den Ende *et al.* (1957). The distribution of CF activity and total N after application of the

original method to rabies infected mouse brain extracts is illustrated by the following example. A crude extract (57 ml., CF titre 1590; 2·63 mg. N/ml.) prepared by Casals method from 120 infected mouse brains was cooled to 4° in a vessel surrounded by ice and provided with a mechanical stirrer and pH meter electrodes. With the temperature adjustment on the meter set at the lower limit of 10°, the reading was adjusted to pH 4·5 by the slow addition of 0·1 N-HCl (about 16 ml.). The suspension remained in ice for 20 min. and was centrifuged for 40 min. at 6600 *g* in the No. 40 rotor with refrigeration. (Later, delay at this stage was avoided.) The supernatant fluid was removed and the precipitate suspended in pH 8·4 saline (15 ml.). (When the supernatant was to be retained it was neutralized at once with Na_2HPO_4). After a night at 4° the suspension was centrifuged at 6600 *g* for 30 min. The supernatant fluid was removed, and the residue extracted twice more with 10 and 5 ml. amounts of pH 8·4 saline, being stirred with the solvent for 1 hr. on each occasion. The three acid-precipitation purified (APP) extracts were combined and the pH 4·5 supernatant fluid and the undissolved residue were sampled for analysis. The APP extract (29 ml.; CF titre 916; 0·52 mg. N/ml.) contained 29 % of the original CF activity with a purity (in terms of total N) 2·9 times that of the crude extract. Of the nitrogen in the crude extract 49 % remained in the pH 4·5 supernatant, 39 % in the residue isoluble at pH 8·4 and 10 % in the APP extract. The CF activity of the (neutralized) pH 4·5 supernatant solution was usually about 5 % of that of the crude extract, and such supernatants were discarded. When, however, the three crude extracts 65/1, 65/2 and 65/3 described above were refined in this way the recoveries of CF activity in the supernatant fluids were respectively 34, 25 and 23 %.

APP extracts were usually dialysed against EDTA saline, centrifuged 1 hr. at 59,000 *g* in the No. 40 rotor (unless the crude extract had received two equivalent centrifugations), dialysed against water and freeze-dried unless required for immediate use. In contrast to the complete recovery of fowl plague soluble antigen from egg membrane extracts achieved by Schäfer & Munk (1952), the recovery of rabies antigens was usually about 30 %. APP extracts, however, appeared to be more stable than crude extracts and nearly all the better results were obtained with them.

Treatment with organic solvents

Emulsification of extracts, crude or APP, with chloroform (van den Ende *et al.* 1957) ether or difluorotetrachloroethane removed all or most of the antigen from the aqueous layer. Part was recoverable with enhanced purity from the emulsion. After emulsification with butanol, however, appreciable activity remained in the aqueous layer. Of these solvents ether appeared to give the best results.

Ether. The freeze-dried residue from 15 ml. APP extract was dissolved in 15 ml. BS containing 'Triton X-100' (0·01 %, w/v) and thiomersalate (0·01 %, w/v). A 0·2 ml. sample (A) was diluted 1/20 for analysis and the remainder cooled in solid CO_2 + acetone mixture until ice began to separate. Intermittent cooling was continued while the solution was shaken vigorously for 10 min. with freshly distilled ether (15 ml.). The mixture was centrifuged in the cold and the lower layer removed and treated twice more with ether (15 ml.) in the same way. The final aqueous layer was partially freed from ether under reduced pressure and a 0·2 ml. sample

(B) was diluted 1/20 in BS for analysis. The gels from the upper phases (that from the third operation being very small) were united with the aid of about 5 ml. of BS and partially freed from ether under reduced pressure leaving a suspension which was dialysed against BS overnight. The suspension was centrifuged 30 min. at 59,000 *g* in the No. 40 rotor, the supernatant fluid removed and the residue suspended in 75 ml. of BS and centrifuged as before. The combined supernatant fluids were diluted to 15 ml. and a 0·2 ml. sample (C) diluted 1/20 with BS. The u.v. absorption curves of the three samples were plotted from 230 to 300 mμ (with an appropriate blank) and 'protein' estimated as already described. The recovery of CF activity was 27 % with a threefold increase in purity based on 'protein' (Table 2). Although all samples absorbed maximally at or near 260 mμ the absorption of the product at this wavelength was only 13 % of that of the starting solution, suggesting that nucleotides as well as 'protein' are partially eliminated by the process.

Table 2. *Ether treatment of APP extract*

Sample	*E*260 mμ	*E*280 mμ	E280/*E*260	CFT/ 'protein'	CF titre	'Protein'
A (original)	0·496	0·292	0·59	21	23	1·1
B (aqueous phase)	0·403	0·210	0·52	1·4	0	—
C (from emulsion)	0·065	0·044	0·63	1·9	6·3	3·3

Table 3. *Batch adsorption with hydroxyapatite*

No. of treatments	*E*260 mμ	'Protein'	CF titre	CF recovery (%)	CFT/ 'protein'	CFT/ *E*260 mμ
0	0·875	32·2	58	100	1·8	66
1	not clear	13·2	40	69	3·0	—
2	0·56	4·9	32	55	6·5	57
3	0·52	4·0	31	53	7·7	60
4	0·46	2·7	26	45	9·6	57
5	0·44	2·8	24	41	8·6	55

Adsorbents

Calcium phosphate (brushite or hydroxyapatite) adsorbed the CF activity and part of the 'protein' from crude or APP extracts in pH 8·2 phosphate buffers at concentrations less than about 0·09 M. Much of the activity was eluted at 0·125 M concentration. Batch and chromatographic methods of purification used before the cellulose ion exchangers were available were based on these observations.

Batch treatment. A suspension of hydroxyapatite (15 ml.) was centrifuged and the packed deposit (5·4 ml.) washed three times with 0·125 M-phosphate pH 8·2 by suspension and centrifuging. The adsorbent was finally resuspended in the buffer and the suspension distributed as evenly as possible between five centrifuge tubes. These were centrifuged, the supernatant fluids removed and the tubes stoppered until required. An extract (32 ml. from 189 infected suckling mouse brains) which had been purified by two successive precipitations at pH 4·5 was dialysed against 0·125 M-phosphate pH 8·2, sampled and stirred for 30 min. at 4° in one of the centrifuge tubes containing the washed adsorbent. The mixture was centrifuged and the supernatant fluid was sampled and treated with fresh adsorbent in the same manner. Stirring, centrifuging, sampling and transference were repeated until the

extract had received five adsorptions. The samples (0·4 ml.) were diluted 1/20 in BS, examined in the spectrophotometer, titrated and used for 'protein' estimation (Table 3). The purity (CFT/'protein') was increased by a factor of 4·8 but substances absorbing at 260 mμ were not selectively removed. The final supernatant fluid from this experiment was treated with ether as previously described, 87 % of the CF activity being recovered. During the ether treatment there was a slight decline (to 6·1) in the purity calculated on 'protein' but the ratio CFT/E 260 mμ was increased by a factor of 6. This removal (in the aqueous phase) by the ether process of substances absorbing at 260 mμ was not always observed but on some occasions the absorption peak was changed from 260 mμ to about 275 mμ.

Chromatography. In an experiment using stepwise elution with pH 8·2 phosphate buffers of increasing concentration from 0·01 M to 0·125 M, 23 % of the CF activity of an APP extract was recovered in two peaks corresponding to the 0·11 M and 0·125 M levels. In the fractions of highest titre the purity (CFT/mg. N/ml.) was 6·3 times that of the extract applied to the column.

Diethylaminoethyl (DEAE) cellulose. The antigen behaved on this adsorbent (which formed more freely flowing columns than calcium phosphate and appeared to have a higher capacity) in a manner closely resembling its behaviour on calcium phosphate, but a higher salt concentration was needed for elution. A crude extract (40 ml.) prepared from about 110 infected brains was dialysed against 0·005 M-phosphate pH 8·2 and applied to a column containing 10 g. of buffer-equilibrated DEAE cellulose. Stepwise elution was carried out with 200 ml. volumes of phosphate mixtures at pH 8·2 of 0·005, 0·09 and 0·125 molarity followed by 0·125 M-phosphate + 0·1 N-NaCl and finally a mixture (pH 6·0) of Na_2HPO_4 and NaH_2PO_4 molarity 0·1 with NaCl 0·5 M. The effluent was collected in 5 ml. fractions. About half of the protein applied to the column appeared in the fraction eluted with 0·09 M buffer which, however, contained negligible CF activity. Fractions eluted with the two most concentrated buffers were assembled in pools which were analysed for 'protein' and CF activity. The total recovery of CF activity was 33 % and the purity (CFT/'protein'), was about three times that of the crude extract. A mixture (303 ml.) of the CF active pools from this experiment was dialysed briefly against water, thoroughly against 0·005 M-phosphate pH 8·2 and applied to a column (13 mm. diam.) prepared from 1 g. of equilibrated DEAE. Stepwise elution was carried out (Fig. 3) and one hundred 2·5 ml. fractions were collected. The CF titre of the influent solution was 19·2 and that of the most active fraction 500. Of the 'protein' applied 93 % was recovered including 13 % in the NaOH solution used to regenerate the adsorbent. Unfortunately 40 % of the initial 'protein' was associated with the fractions containing the CF activity so that the most active fractions were only about twice as 'pure' as the solution applied. The recovery of CF activity was about 40 %.

As the rather striking 'peaks' of protein concentration and CF activity were obviously artifacts attributable to the discontinuous increases in buffer concentration, linear-gradient elution was used in subsequent experiments. An extract (74 ml. CFT 273) which had been purified by two precipitations at pH 4·5, was dialysed against 0·005 M-phosphate pH 8·2 and applied to a column of DEAE cellulose (2 g.) equilibrated with this buffer. The column was rinsed with the 0·005 M buffer until 89 ml. of filtrate containing a negligible amount of protein and no detect-

able CF activity had been collected. Elution was continued with 0·09 M-phosphate pH 8·2 until nine 20 ml. fractions had been collected. Thereafter a linear gradient prepared from 250 ml. of the 0·09 M buffer and 250 ml. of 0·3 M-potassium phosphate mixture pH 6 was passed in and the effluent was collected in 20 ml. fractions. The results (Fig. 4) suggest that there may be four components having CF activity of which two are only very slightly separated. The recovery of CF activity was 60 %

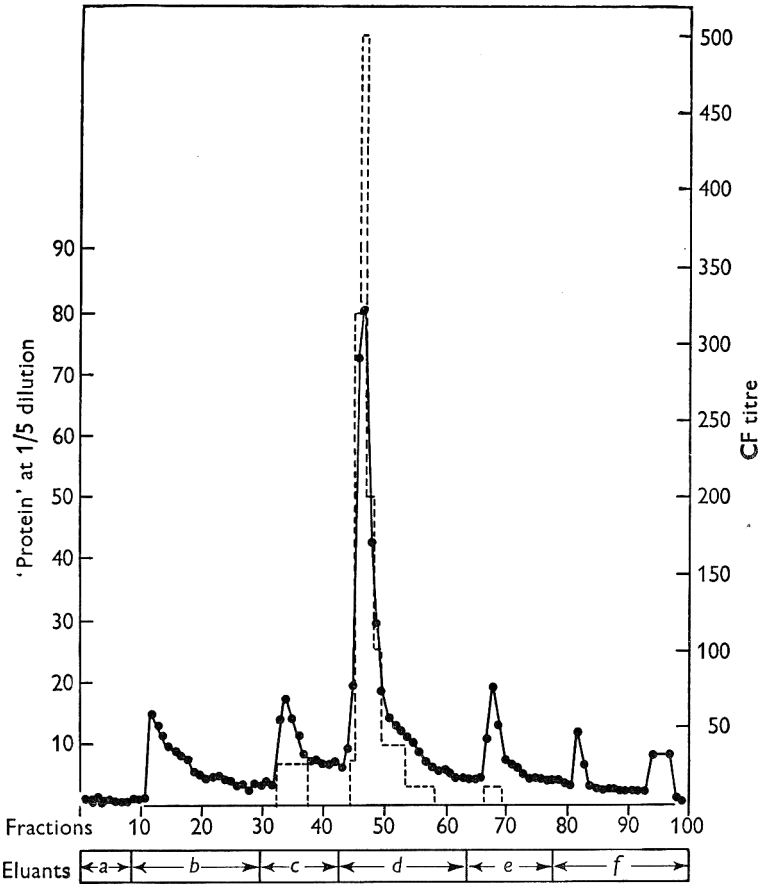


Fig. 3. Repeated chromatography on DEAE cellulose of CF-active fractions from the chromatography of a crude extract. Broken line, histogram of CF titres; full line 'protein' concentrations. Elution was stepwise with the following solutions: (*a*) 0·005 M-phosphate pH 8·2; (*b*) 0·09 M-phosphate pH 8·2; (*c*) 0·125 M-phosphate pH 8·2; (*d*) 0·125 M-phosphate 0·1 M-NaCl pH 8·2; (*e*) 0·125 M-phosphate 0·2 M-NaCl pH 8·2; (*f*) 0·1 M-phosphate 0·5 M-NaCl pH 6.

but the purity improvement factor (less than 2 in the main fraction) was disappointing. Fractions 1–6 were mixed to give pool A and fractions 13–37 to give pool B. 20 ml. portions of A and B were separately dialysed and freeze-dried. The residues, dissolved in very small volumes of CFT saline, were applied to a micro-Ouchterlony plate with mouse anti-rabies serum VI and concentrated rabbit antinormal mouse brain γ-globulin as sources of antibody. The only lines of precipitation detected

were one between B and the mouse serum and two between A and the rabbit γ-globulin. The remainder of B, after dialysis against 0·005 M-phosphate pH 8·2 occupied 390 ml. and had a CF titre of 50. This was applied to a column prepared from 2 g. DEAE cellulose and followed first by 0·005 M buffer until two 20 ml. fractions had been collected and then by a linear gradient prepared from 300 ml. 0·005 M and 300 ml. 0·3 M buffers. The results (Fig. 5) confirmed that 2 or possibly 3 CF active components may have been present in pool B, but also indicated that most of the 'protein' was associated with the active fractions, making it seem unlikely that much further purification could be achieved by this process.

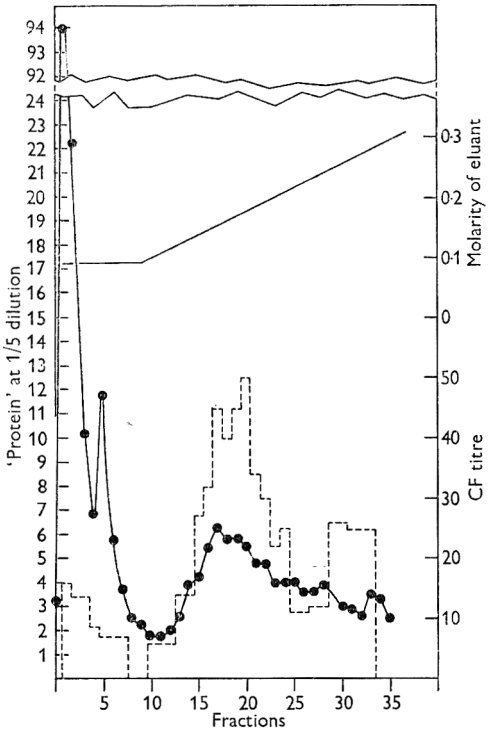


Fig. 4

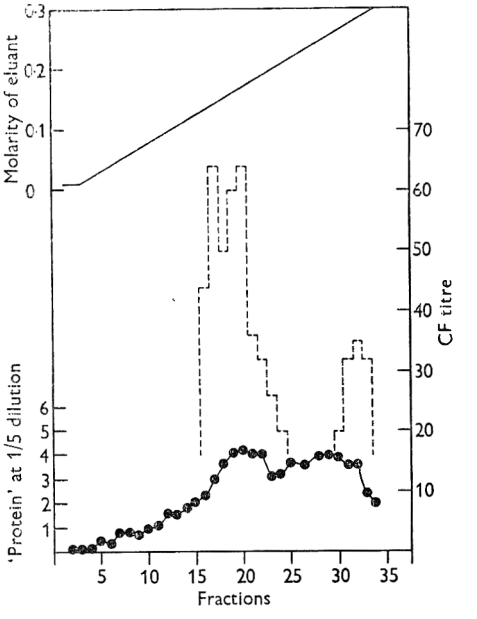


Fig. 5

Fig. 4. Chromatography of APP extract on DEAE cellulose with gradient elution. Broken line, histogram of CF titres; full line and circles, 'protein' concentration; full line without points, elution gradient.

Fig. 5. Repeated chromatography of fractions 13–37 from the experiment shown in Fig. 4. Broken line, histogram of CF titres; full line with circles, 'protein' concentrations; plain line, elution gradient.

Complement-fixing titrations are subject to so many possibilities of error that little importance was at first assigned to the twin peaks (at fractions 16 and 20 in Fig. 4 and fractions 17 and 20 in Fig. 5), but double peaks were later repeatedly observed and are probably significant. Fractions 18 (CFT 50) and 33 (CFT 32) were separately dialysed and dried from the frozen state. The residues dissolved in 0·05 ml. portions of CFT saline were applied to a micro-Ouchterlony plate with

mouse rabies antiserum VI as source of antibody. Only fraction 33 reacted visibly and only one line of precipitate was detected.

Carboxymethylcellulose at the lowest pH (about 6) at which precipitation could be avoided failed to adsorb the CF activity.

Electrophoresis

Starch gel. As the starch gels employed by Smithies (1955) appeared to exert some fractionating effect in addition to that induced by electrophoresis, some of the earliest experiments were made by this technique in the hope that the relatively large antigen would be retained in or near the starting trough while other brain constituents would migrate into the gel. Although this was so, the method was abandoned in the hope that more convenient techniques capable of treating larger quantities of extract would be discovered. Starch gel electrophoresis did, however, confirm the expected complexity of the extracts. In one experiment eighteen bands were detected in a gel slice stained with naphthalene black.

Paper, glass fibre, and cellulose acetate strips. Attempts to control fractionation by paper electrophoresis of extracts before and after chromatography or other treatment were unsuccessful owing to adsorption by the paper. Stained electrophoresis strips usually revealed a narrow intense band at the starting line with a rather evenly stained tail in the direction of the positive electrode, the length of the tail being proportional to the amount of substance applied. Similar results were obtained on glass fibre paper. Cellulose acetate strips (Kohn, 1957) gave better results; two major and about three minor peaks could be detected by a crude scanning photometer. There was, however, too much diffuse staining between these peaks for our purpose.

Agar. In experiments at pH 7·6, pH 8·2 and pH 8·6 crude extracts gave agar electrophoretograms in which 6–9 fractions could be detected; of these, 2 possessed greater mobilities than the albumin fractions of human or mouse serum run simultaneously. Traces of these components were also visible in the mouse serum electrophoretogram. The most intense spots corresponded in position with haemoglobin and albumin. There was much 'tailing' on the negative side of the starting trough at pH 7·6 and on the positive side at pH 8·6. APP extracts gave only a tail on the positive side in which no discrete fractions were detectable. This behaviour accords with the results of immunoelectrophoretic experiments (Mead, 1962).

Starch grains. A preliminary experiment indicated that neither the protein nor the CF activity of a chromatographically purified extract was adsorbed significantly by washed starch grains in pH 6·2 phosphate ($\mu = 0{\cdot}05$). 2 ml. each of fractions 46 and 48 (CFT originally > 200) and about 1 ml. of fraction 47 (CFT originally 500) from the chromatography experiment described above (Fig. 3) were combined, dialysed and freeze dried. The residue was dissolved in 0·5 ml. pH 6·2 phosphate ($\mu = 0{\cdot}05$) and 0·4 ml. injected along the transverse centre line of a slab prepared from 80 g. of washed, ethanol-extracted starch which had been washed twice with the buffer and allowed to drain in the electrophoresis trough. Electrophoresis was continued for 4·5 hr. at 10 V./cm. The current was 21·5—22 mA. and the temperature of the cooling water 11°–12·5°. The positive end of the slab was cut into twenty-four 0·5 cm. sections which were extracted with 5 ml. portions of buffer. Fractions were numbered from the origin and those numbered 8, 11, 13, 15 and 20 separately

titrated. Titrations were also made on pools made by mixing equal volumes of certain fractions. In the best fractions of the twice-chromatographed material there was much protein closely resembling but not coincident with the antigen in mobility (Fig. 6), indicating that further purification by either method was unlikely to succeed.

Columns of modified cellulose. Electrophoresis experiments were done in the modified LKB apparatus to ensure maintenance of constant current and pH during long runs. In an experiment with *crude* extract (Fig. 7) in phosphate at pH 6·0

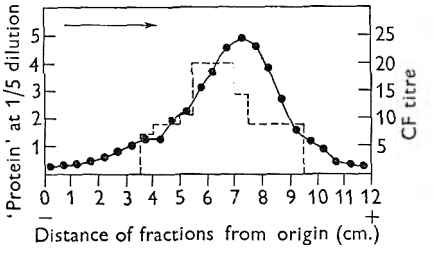


Fig. 6

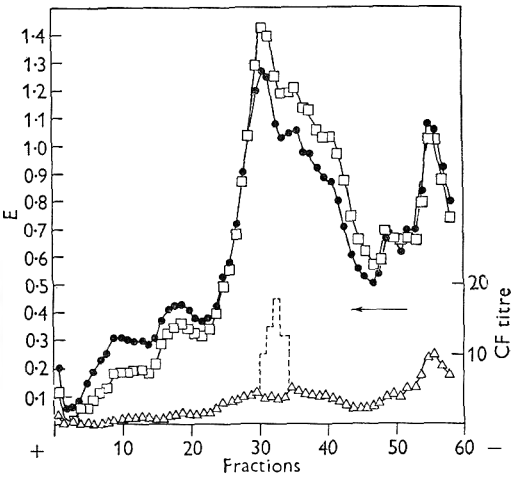


Fig. 7

Fig. 6. Electrophoresis, on starch grains, of the most active fractions from the rechromatography of crude extract on DEAE cellulose (Fig. 3). Broken line, histogram of CF titres; full line, 'protein' concentration. The arrow indicates the direction of migration.

Fig. 7. Electrophoresis of a crude extract in the L.K.B.-Produkter apparatus. —●— absorption of fractions at 260 mμ; —□— absorption at 275 mμ; —△— absorption at 540 mμ; - - - histogram of CF titres. The arrow indicates direction of migration. Absorption at 260 mμ ('nucleotide') exceeded absorption at 275 mμ ('protein') in the first two peaks and even in the last which as indicated by its colour and absorption at 540 mμ was rich in haemoglobin or haemoglobin-like pigment. Separation of red pigment into several fractions was noticed also in chromatograms of crude extracts.

(μ = 0·1) only about 10 % of the CF activity was recovered in a single rather sharp peak with a mobility slightly less than that of the main u.v. (275 mμ) peak but containing a high proportion of the u.v. absorbing material applied to the column. Runs with APP extract were made at pH 5·95 and 8·6 with recoveries of 13 and 36 %. In both runs the peak of CF activity emerged just on the low mobility side of the main protein peak, the separation appearing to be better at the lower pH. The best result was obtained with material purified as follows: A brain extract prepared by the Casals method (with chloroform + methanol extraction) was purified by precipitation at pH 4·5 and refined by two batch treatments with calcium phosphate in 0·125 M-phosphate pH 8·2. The refined extract (30 ml.) was pervaporated, dialysed against water, again pervaporated and dialysed against the electrophoresis buffer (phosphate of pH 6·2 and μ = 0·1 containing 0·01 % (w/v) each of

'Triton X-100' and chloromycetin). The extract (3 ml. CF titre 2600) was applied to the column and displaced downwards with about 10 ml. of buffer. Electrophoresis at 277–280 V, 45–46 mA and 8·5°–8·7°, was maintained for 23 hr. The column fluid was displaced by buffer and collected in 3 ml. fractions which were diluted 1/10 with BS for 'protein' estimation. Those fractions giving a 'protein' reading exceeding about 0·7 were tested for CF activity at 1/10 and those fixing complement

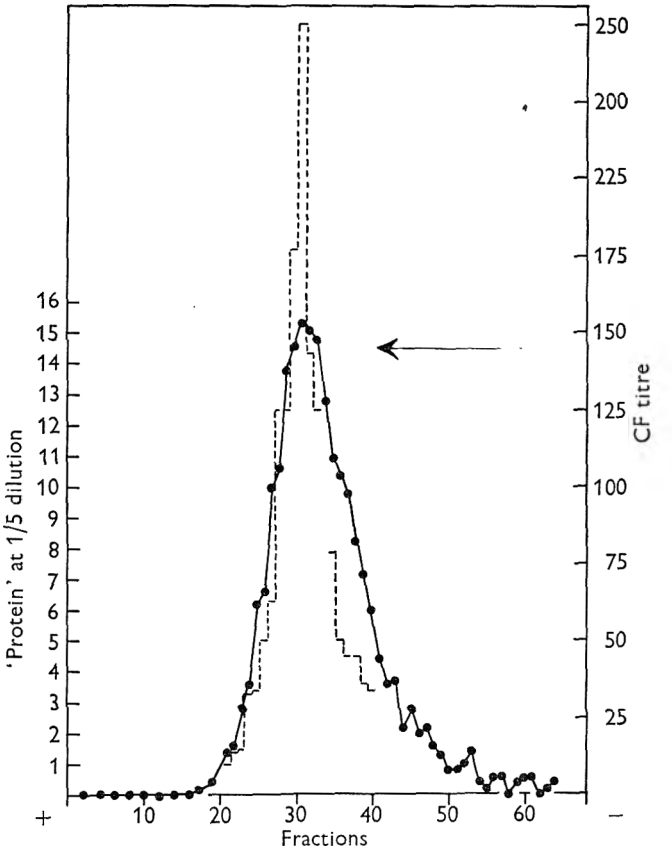


Fig. 8. Electrophoresis of purified APP extract in the L.K.B.-Produkter apparatus. Broken line, histogram of CF titres (no value for fraction 34 was available); full line, protein concentrations. The arrow indicates the direction of migration.

at this dilution were titrated. The recovery of CF was about 60 % but the purity of the best fraction was only about one and a half times that of the starting solution The mobilities of the CF activity and main protein component coincided closely (Fig. 8).

Centrifugation

At this time the antigen was thought to consist only of particles about 12 mμ in diameter (Polson & Wessels, 1953) which might be expected to sediment slowly in the preparative ultracentrifuge. The purest available sample—fractions 22–40 from the column electrophoresis experiment described above (Fig. 8)—was dialysed

against water, pervaporated and dialysed against buffered saline containing 0·01 M-Ca^{++} and 0·01 M-Mg^{++} which were thought likely to promote aggregation. The solution (2·8 ml.) was centrifuged 1 hr. at 6600 *g* in the No. 40 rotor yielding a clear supernatant fluid and a small pellet which was resuspended in 2·8 ml. of the buffer (fraction A). The supernatant fluid was placed in a clean tube, overlaid with the buffer to a total volume of 10 ml. and centrifuged in the No. 40 rotor for 5 hr. at 63,000 *g* to yield a supernatant fraction (B) and a small pellet which was resuspended in 1 ml. of the buffer (C). The three fractions were tested for CF titre and 'protein' content. Of the CF activity recovered, 86 % remained in supernatant B and 14 % was in the pellet (C). Of the initial 'protein' 2 % appeared in A, 84 % in B and 14 % in C. Neither the expected sedimentation of antigen nor any separation of antigen from 'protein' was accomplished. In a similar experiment, APP extract after clarification at 6600 *g* was spun for 6 hr. at 70,000 *g* in the S.W. 39 rotor. The pellet contained 15 % of the initial CF activity and 17 % of the initial 'protein'.

Miscellaneous experiments

CF activity did not appear to be precipitable by streptomycin, a reagent which has been used to purify nucleoprotein particles from a variety of sources (Cohen, 1947; Takata & Osawa, 1957). Four portions of a solution of APP extract were treated with graded amounts of a solution of salmine sulphate while a fifth portion served as control. The mixtures, except the control, contained precipitates and after a night at 4 ° were centrifuged. The supernatant fluids were titrated for CF activity which was found to have been reduced (finally to 18 %) in proportion to the amount of protamine added. In confirmation of van den Ende *et al.* (1957), it was found that only part of the CF activity of extracts was destroyed by digestion with crystalline trypsin at a concentration of 0·2 mg./ml., pH 8·2 at 37° for 30–50 min.

Extracts of normal mouse brains

Several batches of brains from uninfected mice 7–8 days old were extracted and the extracts subjected on various occasions to dialysis precipitation, acid precipitation, calcium phosphate refining, ether treatment and electrophoresis on cellulose acetate strips. Extracts from normal brains (diluted 1/10) did not fix complement in the presence of mouse anti-rabies serum and, except at one of several identical locations in each of two very early experiments, formed no lines of precipitation with this serum in the Ouchterlony test. The elution diagram (from DEAE cellulose) of the protein in the supernatant fluid from pH 4·5 precipitation of normal brain extracts differed slightly from those obtained with pH 4·5 supernatant fluids from infected brains. Otherwise no obvious difference was detected between the behaviour of extracts of normal or infected brains in any of the procedures mentioned.

DISCUSSION

It was not anticipated that purification of the rabies soluble antigen discovered by Polson & Wessels (1953) would be easy. The failure of previous workers to obtain a positive gel precipitin test suggested that the concentration of antigen was small, and the complexity of the tissue extracts implied that specific immunological methods of purification might be needed. It seemed best, however, to remove as

much of the irrelevant brain constituents as possible before attempting purification by specific precipitation. During this preliminary work it was noticed that the complement-fixing components in the brain extracts were unstable and readily adsorbed by precipitates. In attempts at deproteinization by standard methods the CF activity was taken out in the protein fraction but could sometimes be recovered therefrom with its purity improved.

The most effective initial step appeared to be precipitation at pH 4·5. This involved a loss of 50–75 % of the CF activity but the product was more stable. No method of purification with a yield greater than about 50 % was discovered and it was difficult to increase the purity ratio CF activity/concentration of impurities because the numerator diminished during manipulations and short periods of storage about as quickly as the denominator could be reduced by chemical or physical operations.

Batch adsorption of impurities from APP extracts on calcium phosphate under conditions (of buffer concentration) in which CF activity remained in the supernatant fluid, was a fairly efficient method of purification; the product gave a single electrophoresis peak coincident with the CF activity (Fig. 8). Chromatography on calcium phosphate or DEAE cellulose was also useful. Electrophoresis of crude and APP extracts without prior treatment with adsorbents gave peaks of CF activity overlapped by, but not coincident with, a major protein peak (Fig. 7). The protein remaining after treatment with calcium phosphate resembled the CF activity so closely in its electrophoretic behaviour that further purification by methods depending on surface charge seemed likely to prove difficult. Much of the impurity resembling nucleic acid in its u.v. absorption could sometimes be eliminated by treatment with ether when the CF activity entered the gel and the nucleic acid-like impurities remained in the aqueous phase.

The purity of the best samples prepared by these methods was only from two to three up to about ten times that of the crude extract. The absence of visible interaction in the Ouchterlony test between some of the purified fractions and anti-normal mouse brain γ-globulin is attributed rather to the poor antigenic response of the immunized rabbits than to the purity of the rabies antigen.

The author is grateful to Miss I. Staarmann for excellent technical assistance, to Mr G. S. Turner for the neutralization test, to Mr W. H. Borret and Mr K. J. Kaufman for making the fraction collectors and other apparatus, and to his colleagues in the Virus Unit for valuable advice.

REFERENCES

CASALS, J. (1949). Acetone–ether extracted antigens for complement fixation with certain neurotropic viruses. *Proc. Soc. exp. Biol., N.Y.* **70**, 339.

CASALS, J. & OLITSKY, P. K. (1950). A complement-fixation test for poliomyelitis virus. *Proc. Soc. exp. Biol., N.Y.* **75**, 315.

COHEN, S. S. (1947). Streptomycin and desoxyribonuclease in the study of variations in the properties of a bacterial virus. *J. biol. Chem.* **168**, 511.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 2.

FLODIN, P. & KUPKE, D. W. (1956). Zone electrophoresis on cellulose columns. *Biochim. biophys. Acta*, **21**, 368.

FLYNN, F. V. & DE MAYO, P. (1951). Micro-electrophoresis of protein on filter paper. *Lancet*, ii, 235.

GRABAR, P. & WILLIAMS, C. A. JUN. (1955). Méthode immuno-électrophorétique d'analyse des mélanges de substances antigéniques. *Biochim. biophys. Acta*, **17**, 67.

KOHN, J. (1957). A cellulose acetate supporting medium for zone electrophoresis. *Clin. chim. Acta*, **2**, 298.

KOHN, J. (1958). Small scale membrane filter electrophoresis and immuno-electrophoresis. *Clin. chim. Acta*, **3**, 450.

KOHN, J. (1959). A simple method for the concentration of fluids containing protein. *Nature, Lond.* **183**, 1055.

MAYER, M. M., OSLER, A. G., BIER, O. & HEIDELBERGER, M. (1946). The activating effect of magnesium and other cations on the haemolytic function of complement. *J. exp. Med.* 84, 535.

MCKENZIE, H. A. & WALLACE, H. S. (1954). The Kjeldahl determination of nitrogen: A critical study of digestion conditions—temperature, catalyst and oxidizing agent. *Aust. J. Chem.* **7**, 55.

MEAD, T. H. (1962). The characterization of rabies soluble antigens. *J. gen. Microbiol.* **27**, 415.

OSTER, G. (1950). Two phase formation in solutions of Tobacco Mosaic Virus and the problem of long range forces. *J. gen. Physiol.* **33**, 445.

PAIGEN, K. (1956). Convenient starch electrophoresis apparatus. *Analyt. Chem.* **28**, 284.

PARR, C. W. (1954). The separation of sugars and of sugar phosphates by gradient elution from ion-exchange columns. *Biochem. J.* **56**, xxvii.

PETERSON, E. A. & SOBER, H. A. (1956). Chromatography of proteins. I. Cellulose ion-exchange adsorbents. *J. Amer. chem. Soc.* **78**, 751.

POLSON, A. & WESSELS, P. (1953). Particle size of soluble antigen of rabies virus. *Proc. Soc. exp. Biol., N.Y.* **84**, 317.

PORATH, J. (1957). Some cellulose ion exchangers of low substitution and their chromatographic application. *Ark. Kemi*, **11**, 97.

SCHÄFER, W. & MUNK, K. (1952). Reinigung und Eigenschaften eines löslichen Antigens der klassischen Geflügelpest. *Z. Naturf.* 7*b*, 573.

SCHEIDEGGER, J. J. (1955). Une microméthode de l'immuno-électrophorése. *Int. Arch. Allergy, Basel*, **7**, 103.

SMITHIES, O. (1955). Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629.

TAKATA, K. & OSAWA, S. (1957). Ribonucleoprotein from rabbit appendix microsomes. *Biochim. biophys. Acta*, **24**, 207.

TISELIUS, A. (1954). Chromatography of proteins on calcium phosphate columns. *Ark. Kemi*, 7, 443.

TISELIUS, A., HJERTÉN, S. & LEVIN, Ö. (1956). Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* **65**, 132.

URIEL, J. & GRABAR, P. (1956). Emploi de colorants dans l'analyse électrophorétique et immunoélectrophorétique en milieu gélifié. *Ann. Inst. Pasteur.* **90**, 427.

VAN DEN ENDE, M., POLSON, A. & TURNER, G. S. (1957). Experiments with the soluble antigen of rabies in suckling mouse brains. *J. Hyg., Camb.* **55**, 361.

J. gen. Microbiol. (1962), **27**, 415–426
Printed in Great Britain

The Characterization of Rabies Soluble Antigens

By T. H. MEAD
The C.S.I.R. and U.C.T. Virus Research Unit, Department of Pathology, University of Cape Town, South Africa

(*Received* 28 *April* 1961)

SUMMARY

Virus-free extracts of rabies-infected suckling mouse brains contain a relatively large antigen which is slowly sedimentable at 70,000 *g* and resistant to trypsin; a smaller antigen which is destroyed by trypsin; probably at least two antigens of intermediate size also probably sensitive to trypsin. The antigens differ but slightly from each other and from one or more brain components in electrophoretic mobility in agar and in chromatographic behaviour on DEAE cellulose.

INTRODUCTION

Virus-free extracts of rabies-infected suckling mouse brains fix complement in the presence of mouse anti-rabies serum (Polson & Wessels, 1953). Methods for the partial purification of this complement-fixing (CF) activity have been described by Mead (1961). The best preparations retained impurities which closely resembled the specific antigens in electrophoretic mobility, chromatographic behaviour on diethylaminoethyl (DEAE) cellulose and rate of sedimentation in the centrifuge. It therefore seemed likely that further purification by methods dependent on surface charge or sedimentation velocity would be difficult and consideration was given to possible immunological methods.

Sera were prepared in rabbits against normal mouse brains. The antibodies were concentrated by separating the γ-globulin fraction and efforts were made to devise a method for presenting the normal mouse brain antigens existing as impurity in the soluble antigen extracts with their antibodies under optimal conditions for their precipitation and removal (Mead, 1958). At the same time the infected brain extracts were tested against mouse anti-rabies serum by the Ouchterlony technique. At first only one line of precipitation was seen but when a later batch of antiserum was used and especially when this was concentrated twofold, these tests showed that the extracts contained at least two and probably four specific antigens.

METHODS

The virus, preparation of antisera, CF titration, chemicals and buffers, methods of concentration, the preparation of brain extracts, the estimation and significance of 'protein' concentration, methods for chromatography and immuno-electrophoresis and the micro-Ouchterlony technique used have all been previously described (Mead, 1962).

Mouse antisera. Mouse anti-rabies serum batch VIII differed materially from the other batches (VII and IX) used for most of this work. All batches of antiserum were obtained from groups of about 200 adult mice which received ten to thirteen 0·2 ml. intraperitoneal injections of a 10 % emulsion of infected suckling mouse brains at 3 to 4-day intervals. Only the emulsion used for the first injection was normally inactivated with formalin (0·35 %). In the preparation of batch VIII, however, every inoculum was treated in this way.

RESULTS

The number of rabies-specific soluble antigens

Four lines of specific precipitation were seen on several, but not all, occasions on which a concentrated crude extract was tested with anti-rabies mouse serum, batch VII, especially when this serum was concentrated by freeze drying and dissolving the residue in half the initial volume of water (VII $\times 2$). These lines formed rather uniform patterns consisting of an outer, usually rather hazy line near and concave towards the antigen well, a more sharply defined line near and concave towards the antiserum well and two faint narrow and straight lines between these. One of these intermediate lines was often missing and one (not necessarily the same one) was always missing in tests on acid precipitation purified (APP) extracts (Mead, 1962) which, however, frequently showed three lines. Identity, in the Ouchterlony sense, was demonstrated between the antigens in crude and in APP extracts giving rise to the innermost and the outer lines. Different batches of mouse serum, although each was obtained from about 200 mice, varied in their content of precipitating antibodies directed towards the several antigens. The difference was specially marked with batch VIII which usually precipitated only the 'outer' or 'heavy' antigen giving rise to the line nearest the antigen well. The difference, however, was not absolute as a line of precipitation due to one of the 'intermediate' antigens was observed with serum VIII on several occasions.

The position of the line of a given antigen-antibody system on an Ouchterlony plate depends principally on the diffusion constant of the antigen and, to a lesser extent, on the relative concentrations of antigen and antibody in the initial solutions. Large or slowly diffusing antigens tend to form lines nearer to the antigen well than small rapidly diffusing ones but, with constant antibody concentration, the line also moves nearer the antibody well as the concentration of the responsible antigen is increased. With two antigens of roughly equal size the larger, if its concentration is high relative to that of the smaller, may form a line nearer to the antibody well than its companion. In the case of the rabies antigens, one is known from centrifugation experiments (described later) to be relatively large (12 mμ diameter according to Polson & Wessels, 1953) and has invariably been found (in experiments with trypsin, centrifugation or serum VIII) to form the outer line concave to the antigen well but with a curvature becoming less pronounced as its concentration increases. Although the possibility of mistakes cannot be completely excluded, the general identification of this line with the 'heavy' antigen is probably justifiable. The rapidity with which the inner line was formed (within 3 hr. on occasion) together with its closeness and concavity towards the (central) serum well, suggest that it must be due to a relatively very small particle, smaller perhaps than

γ-globulin. As this inner line was nearly always the most prominent it was also regarded as characteristic of the most abundant of the smaller antigens, and is referred to as the 'inner antigen'. Insufficient is known yet about the origin of the two intermediate lines to warrant definite conclusions about size, relative abundance or identity, but as these lines were usually rather straight and, when detectable, were invariably between the characteristically curved inner and outer lines it seems likely that they arose from antigens intermediate in size.

The hypothesis that the four lines of precipitation imply the presence of four different antigens is subject to several objections, notably the possibilities that some lines were formed by a Liesegang effect or that one antigen was broken by enzyme action or laboratory treatment into smaller antigenic fragments differing in size but each capable of forming a line of precipitation. Attempts were therefore made to confirm the antigenic multiplicity suggested by the Ouchterlony technique.

Attempts to distinguish and separate the soluble antigens

Immunoelectrophoresis. No evidence for the existence of more than one antigen was obtained from many experiments with crude extracts which usually gave a single short arc of precipitate with a focus coincident with or slightly on the positive side of the starting trough. In some experiments the arc appeared split at both ends. In immunoelectrophoretograms run with the same crude extract at four different concentrations on one agar slab, one end of all the arcs of precipitation coincided with the starting trough, while the other end extended towards the positive electrode through a distance which diminished with diminishing concentration. This behaviour would be expected if the antigen was adsorbed by the agar and resembled that of lysozyme (Kaminski, 1955) and fibrinogen (Seligman *et al.* 1957), substances which also appear to give normal lines in the Ouchterlony test. APP extracts behaved similarly except that a second arc of precipitation was usually detectable. The best immunoelectrophoretic evidence for the heterogeneity of the antigens was obtained with pooled and concentrated fractions from DEAE cellulose chromatography of APP extracts. The lines of precipitate indicated the presence in fractions from a chromatographic experiment similar to that described in the next section, of two antigens differing but little in electrophoretic mobility and possibly a third differing in mobility but not in immunological specificity. No 'outer' antigen was detectable in either of these fraction pools on Ouchterlony plates. Another immunoelectrophoretogram obtained from fractions intermediate between those used for the experiment last described showed three antigens immunologically but not electrophoretically resolved. Fractions believed to contain principally the outer antigen or from which the other antigens had been removed by digestion with trypsin gave only very short arcs having one end at the origin. Although the immunoelectrophoretic experiments suggested some heterogeneity, clear evidence for the existence of four separable antigens was not obtained.

DEAE cellulose chromatography. In several experiments crude and APP extracts were chromatographed on DEAE-cellulose with linear gradient elution. Phosphate buffers of pH 8·2 were used in the earlier experiments, but a gradient of sodium chloride in phosphate buffer of constant concentration was also employed (Klemperer & Pereira, 1959). The fractions after analysis for 'protein', were concentrated by

freeze drying (either individually or in small pools) and tested by the micro-Ouchterlony technique, usually against several mouse anti-rabies sera. For example, the freeze-dried APP extract from about 185 mouse brains was dissolved in 0·005 M-phosphate pH 8·2 (22 ml.) and the solution, after dialysis against the same buffer, was applied to a column containing buffer-equilibrated DEAE cellulose (4 g.). Elution was begun with a gradient from 0·005 to 0·25 M-phosphate and 148 10 ml. fractions were collected over this range. A steeper gradient from 0·25 to 0·35 M-phosphate was then applied and a further 50 fractions collected. Alternate fractions from 51 to 199 were tested for CF activity at a dilution of 1/5. Activity was detectable in fractions 52–121 and from 150 to 167. Fractions 1–25, 25–50, and the remainder of those showing CF activity were grouped into pools which were concentrated (by dialysis first against water and then against polyethylene glycol) to volumes of about 0·1 ml. for application to micro-Ouchterlony gels. The results suggested the presence of three antigens not separated by this method. In a later experiment it was shown that the large, trypsin-resistant antigen was eluted in the same range as the others.

Table 1. *Sedimentation of CF activity at about* 70,000 *g in the S.W.* 39 *rotor for* 90 *min.*

Sample	Height of liquid column (cm.)	CF titre	CF titre % remaining	'Protein' at 1/5 dilution
Original fluid	—	165	100	11·5
Supernatant tube A	0·4	89	57	3·5
Supernatant tube B	0·75	100	64	5·0
Supernatant tube C	0·95	126	81	6·2

Centrifugation. In preliminary experiments three different volumes of a suspension of washed 'Hyflo-super-cel' (Hyflo) in a dilute (1–2 %, w/v) solution of sucrose in buffer were centrifuged at about 70,000 *g* in the three tubes of the S.W. 39 rotor. The supernatant fluid was removed and replaced by an antigen solution in the same buffer (without sugar) so as to give columns of solution having different heights in the three tubes. These were again centrifuged at about 70,000 *g*, usually for 90 min., and the three supernatant fluids separately analysed for 'protein', CF activity and, after dialysis and concentration, for the presence of the different antigens using micro-Ouchterlony plates and a variety of mouse antisera. The object of the sugar in the fluid enclosed by the Hyflo was to avoid instability caused by the passage of the denser antigen-containing layer into the less dense buffer. It was clear from these experiments that the antigen responsible for the 'outermost' line (often the only line with serum VIII) could be sedimented at 70,000 *g* leaving the 'inner' antigen in the supernatant fluid. The heavy antigen in these experiments was, however, not usually detected in extracts of the Hyflo layer. Some anomalies were noticed in the Ouchterlony lines of the supernatant fluids. For example, a solution of APP extract in 2·9 ml. 0·1 M-'Tris' buffer of pH 7·6 was layered over columns of Hyflo prepared in three tubes, A, B, and C, as described. The tubes were spun for 90 min. at about 70,000 *g* and the supernatant fluids removed and analysed (Table 1). The remainders of the supernatant fractions and a portion of the original solution were dialysed against water and freeze dried. The residues in minimal volumes of

CFT saline were applied to an Ouchterlony plate. The heavy antigen was detectable only in the original solution with either of the sera. With serum VII, however, the inner lines given by fractions A and C showed only partial identity with the inner line given by the original which appeared to be double. This, and the data for the CF activity remaining in the supernatant fluids, suggested that one of the 'inner' antigens was sedimentable under these experimental conditions.

Digestion with trypsin. Incomplete destruction of CF activity by trypsin was noted earlier (Van den Ende, Polson & Turner, 1957) and confirmed by Mead (1961). Experiments showed that the 'inner' antigens were rapidly destroyed by trypsin and that the surviving outer antigen gave a line of precipitation identical in the Ouchterlony sense with that in the undigested mixture. A crude saline extract from 170 infected brains, after dialysis and centrifugation at 59,000 *g* in the No. 40 rotor was dialysed against 0·1 N-veronal buffer pH 8·2 containing 0·01 M-$CaCl_2$. A sample was removed for 'protein' analysis and CF titration, and a portion of it was dialysed and freeze dried to concentrate it for Ouchterlony testing. In the remainder (125 ml.) at 37° 10 mg. crystalline trypsin were dissolved rapidly by swirling. Samples (0·5 ml.) were taken at 10 min. intervals and immediately diluted 1/25 with ice-cold buffer. One ml. of the diluted sample was added immediately to 3 ml. 8 % (w/v) perchloric acid for 'protein' estimation, and the remainder was treated with an equivalent amount of soyabean trypsin inhibitor and retained for CF titration. After 90 min. a further 10 mg. of trypsin were added, and after 140 min. digestion was stopped by crystalline trypsin inhibitor (20 mg.) and a sample (2 ml.) was dialysed and freeze dried for Ouchterlony testing. During digestion the 'protein' values fell exponentially to 22 % of the initial value. The CF activity fell to 24 % of its initial value in the first 55 min. and remained at 20 % from 75 min. to the end. The digested extract was compared on an Ouchterlony plate with the original extract, a crude extract prepared by the Casals method (Mead, 1962), and an APP extract using both wide and narrow spacing and twice concentrated mouse antiserum batch VII. With 'wide spacing' both the saline extract used for this experiment and the crude Casals extract gave four lines of precipitation, the two centre ones being very faint. The APP extract gave two lines only and the digest only one. With 'narrow spacing' the crude extracts both gave three lines and the APP two. The single line given by the digest coincided with the outermost of those given by the crude and APP extracts.

Acid precipitation, centrifugation, trypsin digestion and chromatography

The following scheme for the separation and purification of the 'inner' and 'outer' antigens was based on the finding that much of the 'protein' in APP extracts was sedimentable with the outer antigen at 70,000 *g*. The inner antigen was thus purified, while the protein, concentrated into the deposit with the outer antigen, could be removed by subsequent treatment with trypsin.

The saline extract (300 ml.) from 187 infected suckling mouse brains was centrifuged for 2 hr. at 44,000 *g* in the No. 30 rotor and a sample (5 ml.) of the supernatant fluid was dialysed and freeze dried for analysis and Ouchterlony testing. The remainder was subjected to two successive precipitations at pH 4·5 (Mead, 1962), the extract from the first precipitation being centrifuged at 59,000 *g* in the No. 40 rotor for 1 hr. to ensure the removal of virus particles. The dialysed extract was

freeze dried. One-eleventh was retained for Ouchterlony tests (the crude extract gave four and the APP two lines of specific precipitate) and five-elevenths dissolved in 6 ml. 0·1M-'Tris' buffer pH 7·6. The solution was divided among three small tubes in each of which 0·1 g. of washed Hyflo-supercel had been packed at 70,000 *g* from a suspension in the buffer. The tubes were centrifuged in the S.W. 39 rotor for 6 hr. at 70,000 *g*. The supernatant fluid, which contained about 29 % of the initial 'protein' was removed and dialysed against 0·02M-phosphate for the chromatograph experiment (*a*) below. The 'Hyflo' pellets were transferred to one tube with 3 ml. of 0·1 N-veronal pH 8·2 containing 0·01 M-$CaCl_2$ and 1·5 mg. of crystalline trypsin. This suspension was stirred at 37° for 1 hr., left at 4° overnight and centrifuged to remove the 'Hyflo'. The supernatant fluid, which contained about 1·5 % of the original 'protein', was dialysed first against Versenate saline (to avoid precipitation of calcium phosphate) and then against 0·02M-phosphate pH 8·2 for chromatograph experiment (*b*).

(*a*) *Chromatography of the supernatant fraction.* The solution was applied to a column prepared from 0·35 g. DEAE cellulose and eluted with a linear gradient from 0·02M-phosphate pH 8·2 (40 ml.) and 0·02M-phosphate containing 0·7M-NaCl (40 ml.). The effluent was collected in 2·75 ml. fractions which were sampled for 'protein' estimation (Fig. 1, curve A), dialysed and freeze dried. The dry residues dissolved in 0·05 ml. volumes of CFT saline were applied to Ouchterlony plates. Antigen was detectable in fractions 11–24 (corresponding to NaCl concentration range 0·2–0·5M) and appeared to have the highest concentration as judged by the relative proximity of its precipitation line to the antiserum well (on three plates) in fraction 13 appreciably in advance of the main protein peak. With 'wide spacing' only one antigen was detected but with 'narrow spacing' a faint narrow line perceptible just outside the main line from fractions 14–16 indicated the presence of a second antigen at low concentration. Similar results were obtained by chromatography of the 70,000 *g* supernatant fluid from the remaining five-elevenths of the APP extract in exactly the same way, but on TEAE cellulose (Fig. 1, curve B). This adsorbent gave rather more sharply defined protein peaks. Antigen was detectable by the Ouchterlony test in fractions 10–24, and again the lines given by some fractions were double when serum IX was used as antibody source but evidence for the presence of more than one antigen was less satisfactory than in the experiment with DEAE cellulose. The residues of fractions 12–17 from the TEAE cellulose column were combined, dialysed and freeze dried. An Ouchterlony test revealed only one antigen. The solution was centrifuged and the clear supernatant fluid adjusted to about pH 5 with a trace of 3·5 N-acetic acid. A precipitate formed and was centrifuged down. The supernatant fluid was neutralized with sodium phosphate solution. The precipitate was treated with pH 8·4 saline by which it was completely dissolved. The supernatant and precipitate fractions, tested by the Ouchterlony method, gave lines of precipitation which were clearly not identical. The results of the precipitation experiment suggest that the solution applied to the column contained two antigens identical in chromatographic behaviour in concentrations such that their lines of precipitation overlapped. The precipitation at pH 5 may then have so disturbed the relative concentrations of the antigens that both could be detected.

(*b*) *Chromatography of the trypsin-treated deposit fraction.* The digested solution

prepared as described above was applied to a column prepared from 0·2 g. equilibrated DEAE-cellulose, and eluted with a gradient of 0·02 M-phosphate and 0·02 M-phosphate containing 0·7 M-NaCl in exactly the same manner as the supernatant fraction. The fractions gave small turbidities with perchloric acid (Fig. 1, curve C)

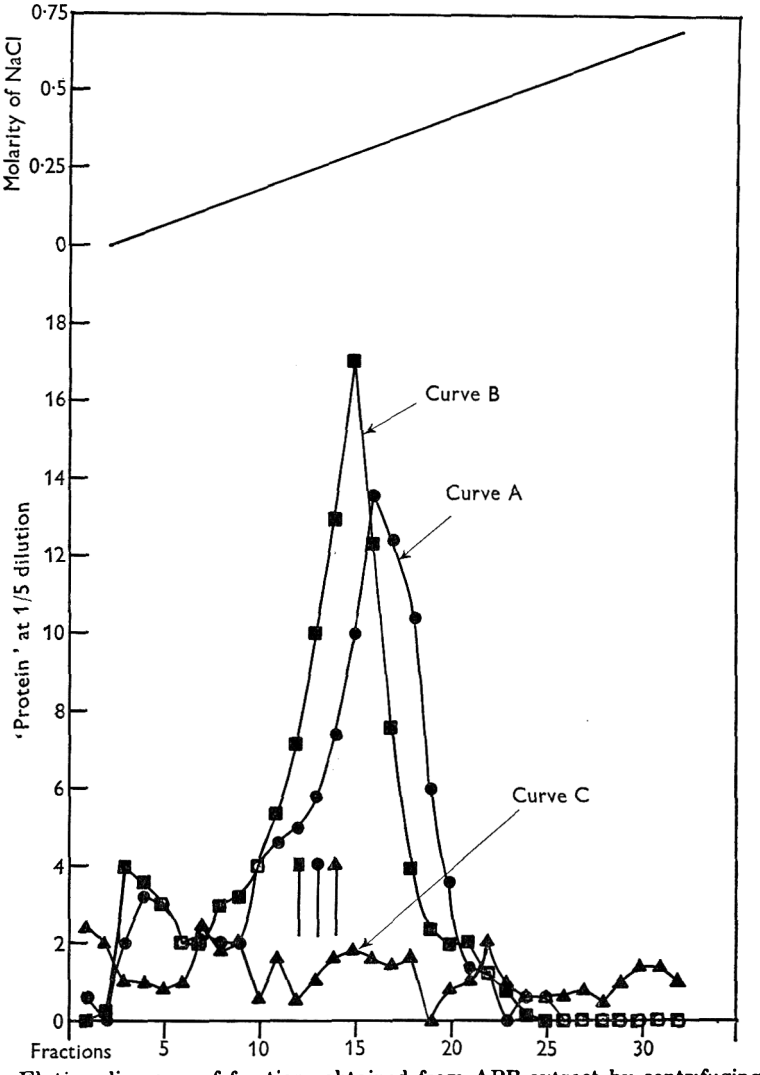


Fig. 1. Elution diagrams of fractions obtained from APP extract by centrifuging and trypsin digestion. —●— Curve A, supernatant fraction chromatographed on DEAE cellulose. —■—, Curve B, supernatant fraction chromatographed on TEAE cellulose. —▲—Curve C, trypsin digested deposited fraction chromatographed on DEAE cellulose. The vertical lines surmounted by the symbols ● ■ ▲ indicate the approximate positions of the antigen elution peaks corresponding to the three curves. The plain line shows the elution gradient.

but no major 'protein' peaks. The fractions were dialysed and freeze dried and the residues, dissolved in 0·05 ml. volumes of CFT saline, were tested on Ouchterlony plates. Fractions 14 and 15 (NaCl concentration 0·26 M) alone gave precipitin lines.

These were, as expected, near and concave to the antigen wells but in two plates appeared to be double. In a parallel experiment in which TEAE-cellulose was used for chromatography under otherwise identical conditions, antigen appeared at the highest concentration in fractions 14 and 15 but could be detected in 16 and 17 as well. On two plates, one with serum VIII $\times 2$ and the other with serum IX $\times 2$ the lines of precipitation presented an unusual appearance (Fig. 2).

Chromatography of the supernatant fluid from pH 4·5 *precipitation.* The early finding that the supernatant fluids from the purification of brain extracts by precipitation at pH 4·5 contained a negligible amount of antigen was based on the use of antisera with which at the most two antigens could be detected by specific precipitation. CF titrations with later batches of antiserum indicated that 23–35 % of the initial activity was sometimes detectable in this supernatant fraction; an attempt was therefore made to detect the more recently discovered antigens in

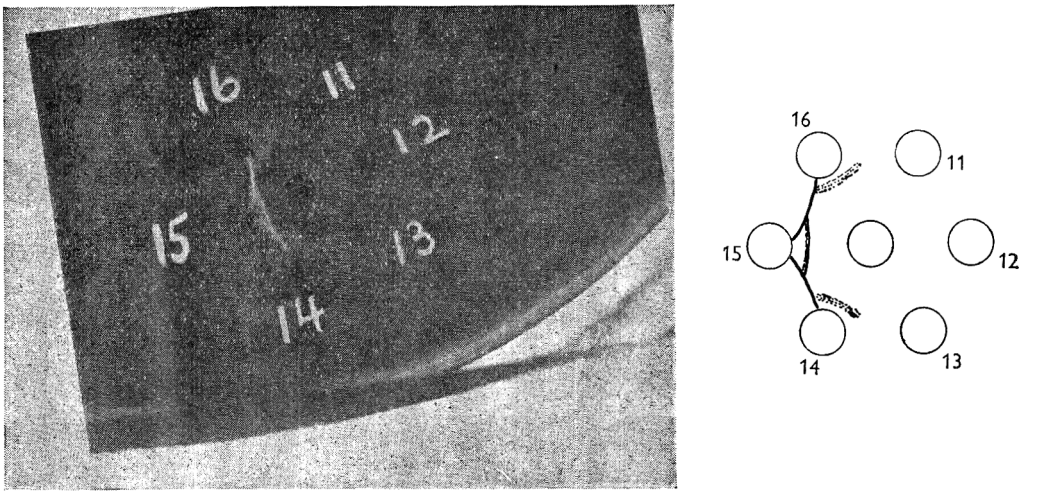


Fig. 2. Unusual appearance of Ouchterlony line given by the 'heavy' antigen purified by sedimentation, trypsin digestion and chromatography. The numbers indicate chromatograph fractions.

these fractions and perhaps separate them by chromatography. The neutralized supernatant fluid (130 ml. CFT 225) from the purification of batch 65/3 (Mead, 1961) was treated with merthiolate (0·01 %, w/v), pervaporated and dialysed first against water and then 0·02 M-phosphate, pH 8·08. The fluid (55 ml.) was run through a column prepared from 3·2 g. DEAE-cellulose and followed by the same buffer until the 'protein' reading of the filtrate had fallen to 2·1 at 1/10 dilution. A linear gradient from 240 ml. 0·02 M-phosphate and 240 ml. 0·02 M-phosphate containing 0·7 M-NaCl was then applied and 10 ml. fractions were collected. The fractions were sampled for 'protein' estimation (Fig. 3, curve A) and pools made by mixing portions of successive foursomes were roughly titrated for CF activity. Fractions forming part of pools with a titre exceeding 20 were then individually titrated (Fig. 3, histogram B). The recovery of CF activity was 50 % and the purities (CFT/'protein') of the three best fractions were 2·2, 2·3 and 3·2, that of the original fluid being 0·44.

One-tenth of each of the fractions 8–19 and the whole remainders of fractions 20–23 were separately dialysed and freeze dried. The residues were dissolved in 0·05 ml. portions of CFT saline and applied to micro-Ouchterlony plates with mouse antisera VII ×2, VIII ×2, IX ×2 and VII + VIII. A strong line of precipitation, apparently common to all fractions from 11 to 24, appeared on all the plates except

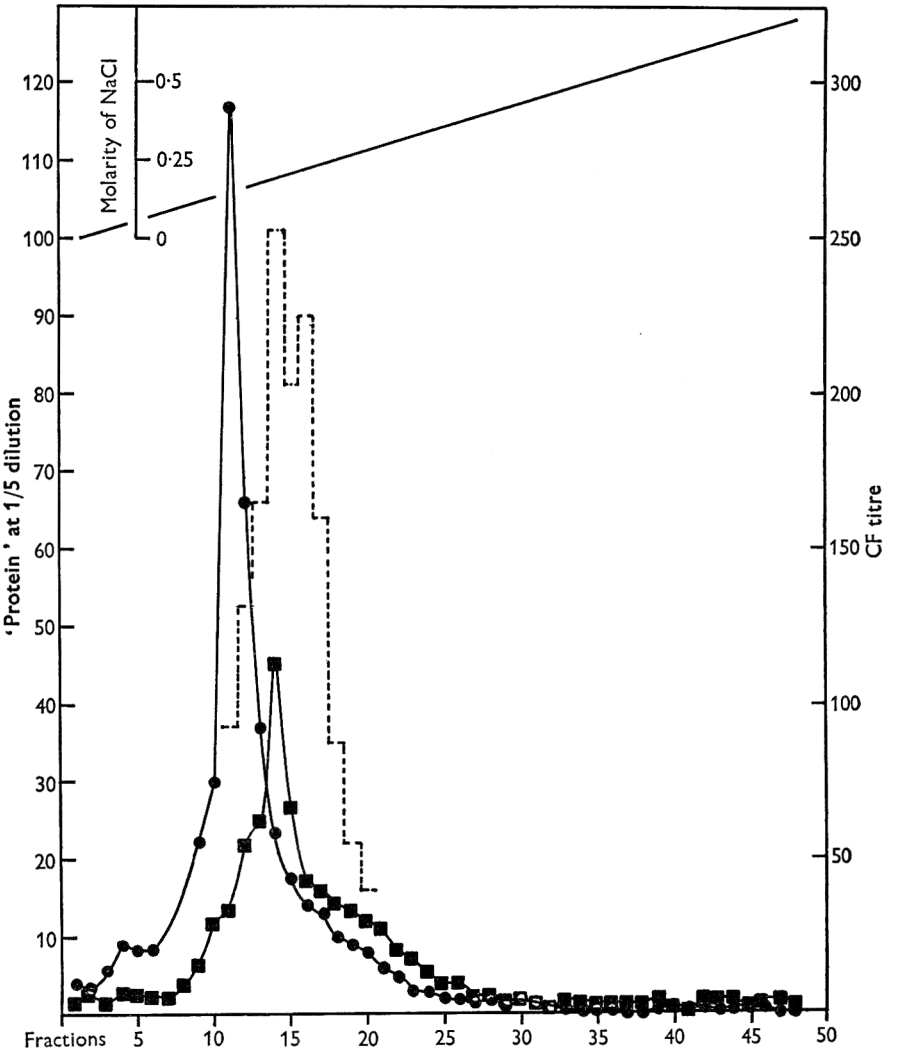


Fig. 3. Elution diagrams of the pH 4·5 supernatant fluids from infected and normal brains. —●— Curve A, 'protein' (infected brains); – – – –, histogram B, CF titres; —■— Curve C 'protein' (normal brains). In contrast to the behaviour of centrifuged APP extracts (Fig. 1) the CF peak follows the 'protein' peak and coincides roughly with the 'protein' peak of the uninfected brains.

that using serum VIII ×2, which gave a faint sharp line extending over the same range but no indication of the presence of the 'outer' antigen. Sera VII + VIII revealed a second line outside the common 'ring' in fraction 17 only, but serum IX ×2 showed two outer lines in fractions 15, 16 and 17. The outermost of these

additional lines was concave to the antigen walls, but probably not due to the 'outer' antigen because this was not detectable with serum VIII. The outer lines were probably not an example of the Liesegang phenomenon caused by excessive antigen because they did not coincide with the highest antigen peak. They did, however, coincide with the second CF peak in the elution diagram.

Of the other two pH 4·5 supernatants having unusually high CF titres, one gave a CFT elution curve with twin peaks and evident, though not so clearly displayed, multiplicity of antigens in the same region, while the other gave a curve with one less pronounced peak and Ouchterlony diagrams in which multiplicity of antigens was not detected. In all the elution diagrams obtained with the pH 4·5 supernatant fluids, the CF activity was eluted at a higher salt concentration than the main 'protein' fraction which emerged at highest concentration in fractions, 11, 11 and 12 in the three experiments. A pH 4·5 supernatant fluid prepared in the same way from an extract of uninfected mouse brains was eluted with a 'protein' peak at fraction 14 (Fig. 3, curve C).

DISCUSSION

Gel precipitin tests in which several lines have arisen from a single antigen have been frequently described (Wilson & Pringle 1954; Grabar, 1957). In some cases extreme excess of one reagent, temperature fluctuations, or refilling of wells, have been blamed for this so-called Liesegang effect. In others, where the appearance of 'extra' lines has followed concentration of the antigen, it is conceivable that one or more unsuspected antigens have thereby been brought up to a concentration at which they formed visible precipitates. Examples of this were encountered here when serial dilutions of an antigen solution were tested against the same antiserum. The 'outer' antigen under these circumstances often gave a precipitate in the first two or three dilutions only, whereas the 'inner' antigen could be detected up to a dilution of 1/16 or more. However, these two particular antigens have been clearly differentiated by their rates of sedimentation, behaviour towards trypsin and precipitability by different antisera, so that the question of the Liesegang effect does not arise. Another possibility is that one or more of the subsidiary lines of precipitation (in this case the lines between those of the 'inner' and 'outer' antigens) may have been due to substances arising from the breakdown (by enzymes or mishandling) of the larger antigen as observed with serum albumin by Lapresle (1955). It could not, therefore, be assumed that every one of the four lines observed corresponds to a different antigen-antibody system and attempts were made to confirm the existence of four such systems by immunoelectrophoresis. Although three lines of precipitation were seen in the immunoelectrophoretogram of a chromatograph fraction, these ran parallel for most of their length and provided no additional evidence for antigenic multiplicity, although the similar mobilities of the antigens accorded well with their chromatographic behaviour. A tendency to retardation of the antigens, especially the largest, by agar is believed to be an additional complication.

If the stability problem can be overcome, purification of the largest antigen should be greatly simplified by its sedimentability and resistance to trypsin. The other antigens, though very similar in their chromatographic behaviour, are probably not identical in this respect, because two (or more) peaks of CF activity have often

been seen in curves obtained by gradient elution suggesting that complete separation of these also may depend on improving the stability and on more refined chromatographic technique.

It was frequently observed that crude extracts giving four lines of precipitation gave only two or occasionally three after purification by precipitation at pH 4·5, the missing line or lines being one or both of the faint ones lying between the main 'inner' and 'outer' ones. It was therefore interesting that the supernatant fluids from two pH 4·5 precipitations gave twin-peaked CF-elution curves on chromatography, and that some fractions gave three lines of precipitation with serum IX but no 'outer' one with serum VIII, indicating that none of the three was due to the 'outer' antigen.

Extracts of normal and infected brains resemble one another closely in their general behaviour. In extracts of infected brains a portion of the 'protein' corresponds closely in electrophoretic and chromatographic properties with the antigens (Mead, 1962). These findings agree with the hypothesis that one or more antigens are normal tissue components that acquire a new immunological specificity during infection without much change in physical properties.

There is little obvious resemblance between the rabies antigens and the three antigens found by Pereira and his collaborators (Klemperer & Pereira, 1959; Allison, Pereira & Farthing, 1959; Pereira, 1960) in cultured tissues infected with adenoviruses. These antigens remain in the aqueous layer on treatment with fluorocarbon, differ markedly from each other in mobility and chromatographic behaviour, and are associated with interesting biological effects.

REFERENCES

Allison, A. C., Pereira, H. G. & Farthing, C. P. (1959). Investigation of adenovirus antigens by agar gel diffusion techniques. *Virology*, **10**, 316.

Grabar, P. (1957). Agar-gel diffusion and immunoelectrophoretic analysis. *Ann. N.Y. Acad. Sci.* **69**, 525.

Kaminski, M. (1955). Studies on egg white and its constituents by immunochemical techniques in gelified media: specific precipitation by double diffusion and immunoelectrophoretic analysis. *J. Immunol.* **75**, 367.

Klemperer, H. G. & Pereira, H. G. (1959). Study of adenovirus antigens fractionated by chromatography on DEAE-cellulose. *Virology*, **9**, 536.

Lapresle, C. (1955). Étude de la dégradation de la sérumalbumine humaine par un extrait de rate de lapin. II. Mise en évidence de trois groupements spécifiques différents dans le motif antigénique de l'albumine humaine et de trois anticorps correspondents dans le sérum de lapin anti-albumine humaine. *Ann. Inst. Pasteur*, **89**, 654.

Mead, T. H. (1958). Preparative immunoelectrophoresis. *Biochem. J.* **69**, 48*P*.

Mead, T. H. (1962). Purification of rabies soluble antigens. *J. gen. Microbiol.* **27**, 397.

Pereira, H. G. (1960). A virus inhibitor produced in HeLa cells infected with adenovirus. *Virology*, **11**, 590.

Polson, A. & Wessels, P. (1953). Particle size of soluble antigen of rabies virus. *Proc. Soc. exp. Biol., N.Y.* **84**, 317.

Seligman, M., Goudemand, B., Jamin, A., Bernard, J. & Grabar, P. (1957). Études immunochimiques sur la présence de fibrinogène dans des extraits de plaquettes humaines lavées et dans certains extraits leucocytaires. *Rev. Hémat.* **12**, 302.

van den Ende, M., Polson, A. & Turner, G. S. (1957). Experiments with the soluble antigen of rabies in suckling mouse brains. *J. Hyg., Camb.* **55**, 361.

Wilson, M. H. & Pringle, B. H. (1954). Experimental studies of the agar-plate precipitin test of Ouchterlony. *J. Immunol.* **73**, 232.

Note added in proof

Three new mouse anti-rabies sera reveal the two intermediate and the outer antigens, but, like VIII, lack antibodies precipitating the 'inner' one. One of these sera was prepared with formalin-treated inocula, but for the others only the first one or two of about twelve inocula were so treated. The cause of the difference in antibody response remains obscure, but it is evidently not exposure to formaldehyde.

J. gen. Microbiol. (1962), **27**, 427–435
Printed in Great Britain

Further Mutational Changes in the Photosynthetic Pigment System of *Rhodopseudomonas spheroides*

By MARY GRIFFITHS*
Biology Department, Lawrence College, Appleton, Wisconsin, U.S.A.

(*Received* 8 *May* 1961)

SUMMARY

Two further groups of pigment mutants have been isolated from irradiated suspensions of the non-sulphur purple bacterium *Rhodopseudomonas spheroides*. Both contain the normal red carotenoid of the wild type, but in place of bacteriochlorophyll one group contains a derivative at the oxidation level of chlorophyll *a*, and the other a pigment at the oxidation level of protochlorophyll. Both these groups are non-photosynthetic. All attempts to produce stable photosynthetic mutants containing a pigment absorbing at the same wavelengths as chlorophyll *a* failed, although transient photosynthesis seemed to occur on several occasions. The relationship of these mutants to the biosynthetic pathway of bacteriochlorophyll suggested by Granick is discussed.

INTRODUCTION

In a previous study of the pigments of the photosynthetic bacterium *Rhodopseudomonas spheroides*, several mutants were isolated which were blocked at different points in the biosynthetic pathway of carotenoids. Investigation of the pigments they accumulated threw some light on the normal mechanism of carotenoid synthesis in the wild type (Griffiths & Stanier, 1956). One of the mutants isolated, which contained normal bacteriochlorophyll, excreted considerable amounts of different bacteriochlorophyll derivatives into the medium (Sistrom, Griffiths & Stanier, 1956). These were analysed, but it was not clear whether they were compounds in the normal biosynthetic pathway of bacteriochlorophyll, or a variety of rather meaningless degradation products. In the present study, further mutants have been isolated; these show blocks in bacteriochlorophyll synthesis. The pigments they accumulate within the cell have been isolated and studied in an attempt to acquire more information about the normal biosynthetic pathway of bacteriochlorophyll.

METHODS

Biological material and media. Rhodopseudomonas spheroides strain 2.4.1, obtained from the collection of Professor C. B. van Niel (Hopkins Marine Station of Stanford University), was the wild-type used as starting material for the production of mutants.

For growth on agar plates, a 1 % (w/v) solution of yeast extract (Difco Labora-

* Present address: Department of Zoology, University of Washington, Seattle 5, Washington, U.S.A.

tories, Detroit, U.S.A.) in tap water was used. For growth in liquid culture, either aerobically or photosynthetically, a partially defined medium described by Cohen-Bazire, Sistrom & Stanier (1957) was used. Non-photosynthetic mutants were grown semi-aerobically, in partially filled Erlenmeyer flasks, in the dark, unless otherwise stated.

Isolation of mutants. The isolation of mutants, produced by ultraviolet irradiation of the wild-type, was described previously (Griffiths & Stanier, 1956).

Isolation and determination of the pigments

Carotenoids. Washed organisms were extracted three times with an acetone + methanol mixture (7 + 2 by vol.) which removed all of the pigments. The carotenoids were extracted from this mixture by means of an equal volume of light petroleum (b.p. 60–70°). The light petroleum extract was washed with the acetone + methanol mixture and then with water, concentrated, and placed on a column of aluminum oxide, activity grade 2 (Fisher Scientific Company, New York). The column was developed with light petroleum and acetone.

Bacteriochlorophyll derivatives. Washed organisms were extracted three times with 80 % (v/v) acetone in water. As this extracts some of the carotenoids, as well as the bacteriochlorophyll derivatives being investigated, the former were removed by addition of an equal volume of light petroleum (b.p. 60–70°) in a separatory funnel. The green lower acetone phase which contained the bacteriochlorophyll derivatives was run off and extracted three times with light petroleum to remove traces of carotenoids. Diethyl ether was then added to the acetone phase and the pigments transferred to the ether by the cautious addition of water. The ether phase was washed with water to remove residual acetone, concentrated, and placed on a sucrose column. The column was developed with an ether + light petroleum mixture (2 + 3 by vol.), and the pigment eluted from the column with ether. All spectra were determined with a Beckman model DU spectrophotometer.

RESULTS

The pigments of the wild-type organism have been described previously. For purposes of comparison it is sufficient to recall that *Rhodopseudomonas spheroides* has three pigments in its photosynthetic apparatus: two carotenoids and bacteriochlorophyll. When the wild-type is grown under non-photosynthetic conditions, semi-aerobically in the dark, the *in vivo* absorption spectrum obtained is that shown in Fig. 1. In living organisms, the region of carotenoid absorption is between 450 and 575 mμ, the remaining peaks at 370, 590 mμ and between 800 and 900 mμ being due to bacteriochlorophyll. When the pigments are extracted into organic solvents, there is a shift in the position and pattern of the long wavelength peaks in the spectrum of bacteriochlorophyll, the complex absorption between 800 and 900 mμ being replaced by a single peak at 775 mμ (Fig. 2).

In the present study, more than 20 mutants showing deranged chlorophyll synthesis were isolated. They fall into two main groups having pigments with long wavelength absorption maxima at about 625 and 660 mμ, respectively, in organic solvents (Fig. 2). They will be referred to as the 625 mutants and the 660 mutants, since it is not possible to distinguish between them on the basis of colour. When

grown on agar plates, all the mutant colonies are of some shade of green, whereas the wild-type is red. The 625 mutants are in general yellower than the 660 mutants, but within each group there is considerable variation in colour, due to differences in the ratios of the amount of carotenoid pigment to the amount of bacteriochlorophyll derivative present.

Figure 1 shows the absorption spectra of young organisms grown aerobically on agar plates in the dark. The organisms were treated for 10 min. in a 9 KC Raytheon sonic oscillator to decrease the scattering when the absorption spectra were determined. It has previously been found on many occasions that this treatment does not alter the position of the peaks in the *in vivo* spectrum. It can be seen that there were

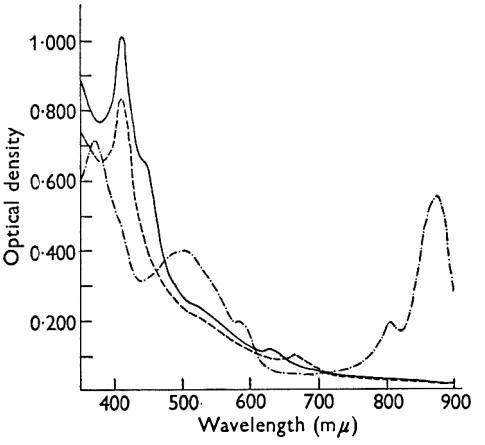


Fig. 1

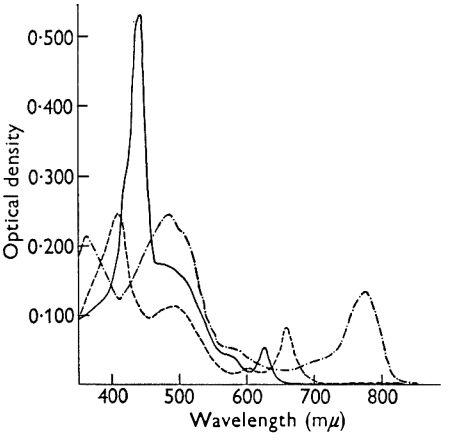


Fig. 2

Fig. 1. Absorption spectra of young *Rhodopseudomonas spheroides* organisms grown semi-aerobically in the dark. The spectra of the 660 mutants (---) and of the 625 mutants (—) are compared with that of the wild type (·····). The organism had been treated in a sonic oscillator for 10 min. to decrease light scattering.

Fig. 2. Absorption spectra of acetone + methanol extracts of organisms grown semi-aerobically in the dark. Wild-type of *Rhodopseudomonas spheroides* (·····); 660 mutants (---) and 625 mutants (—).

several striking changes in the spectra of the mutants compared with that of the wild-type grown under the same conditions. It is not possible from these spectra to say what kind of carotenoids are present in the mutants, but there was still considerable absorption in the region around 500 mμ, suggesting that carotenoids were present. With regard to bacteriochlorophyll absorption, there were radical changes. Both groups of mutants lacked the infrared peaks of the wild-type and the Soret peak at 370 mμ. Instead, the 660 mutants had a long wavelength maximum at 665 mμ, and a short wavelength peak at 410 mμ. The 625 mutants had a long wavelength maximum at 630 mμ, and a short wavelength peak at 412 mμ, with a pronounced shoulder at 440 mμ.

More information about the pigments in the mutants was obtained from their absorption spectra in organic solvents. Figure 2 shows the spectra obtained when organisms of typical mutants from each group were extracted with an acetone + methanol mixture (7 + 2 by vol.). The spectrum of the normal strain, similarly

treated, is also given. In the 625 mutants there was usually a slight shoulder at 415 mμ, a main peak at 440 mμ and a smaller peak at 625–630 mμ, due to bacteriochlorophyll derivatives, and a plateau around 475 mμ in the region of carotenoid absorption. The 660 mutants all had a major peak at about 415 mμ, and a long wavelength maximum at 660–662 mμ due to a bacteriochlorophyll derivative. The greatest variation in this group was in the extent of absorption in the carotenoid region; but all showed a peak or plateau at about 490 mμ. In both groups of mutants the absorption due to carotenoids around 475 mμ was quite like that found in the normal strain grown under the same conditions, but there were striking differences in the positions of the long wavelength maxima due to bacteriochlorophyll derivative absorption: from 775 mμ for the normal strain to 660 and 625 mμ, respectively, for the mutant groups.

Identification of pigments

The two groups of mutants clearly did not contain any bacteriochlorophyll. To determine the nature of the bacteriochlorophyll derivatives, and of the carotenoids present, the pigments were separated by solvent partition and purified by chromatography as described in methods.

Carotenoids. The absorption spectrum of the main pigment found in the 660 mutants was identical with that of the normal red carotenoid found in the wild-type. There was also a trace of a second red pigment which absorbed towards longer wavelengths than the normal red. This pigment has been detected previously in dark red mutants of *Rhodopseudomonas spheroides* (Griffiths & Stanier, 1956), but has not been obtained in sufficient quantities for purification. No yellow carotenoid was found, but none is produced in the wild-type, in any quantity, under aerobic conditions. The carotenoids of the 625 mutants were not purified by chromatography, but the spectrum of the crude carotenoid extract was the same as for the 660 mutants, and it can be safely concluded that the main carotenoid of both groups is the normal red pigment of the wild-type.

Bacteriochlorophyll derivatives. The spectra of the mutant pigments after purification by solvent partition and chromatography are shown in Fig. 3. For each mutant only one chlorophyll derivative was present in appreciable amounts. In determining the nature of these compounds, as far as is possible from the absorption spectra, the positions of the maxima and their relative heights are of importance (Stern & Wenderlein, 1936; Stern & Pruckner, 1939; Rabinowitch, 1951). The position of the long wavelength maximum gives the level of oxidation of the compound, and the relative heights of the smaller peaks indicate the presence or absence of magnesium.

660 *mutants.* The longest wavelength peak was at 660 mμ, a displacement of 115 mμ to shorter wavelengths when compared with bacteriochlorophyll. The highest peak was at 450 mμ with smaller peaks at 470, 502, 531, 555 and 603 mμ. Within the group there were very slight variations both in the positions of the peaks and in the relative heights of the peaks. Differences of the magnitudes encountered can be explained as due to slight differences in the nature of the side groups attached to the molecule. The position of the long wavelength maximum at 660 mμ indicates that the pigment was not at the same oxidation level as bacteriochlorophyll, but at the higher oxidation level characteristic of chlorophyll *a* and its derivatives. The

presence of well defined peaks at 502 and 531 mμ shows that the pigment molecule lacked magnesium and could not be chlorophyll *a* (Rabinowitch, 1951, p. 628). Comparison with published data (Table 1) shows that the spectrum is very similar to that found by Smith & Benitez (1955) for pheophytin *a*, which is chlorophyll *a* with the magnesium removed, and even closer to that of a Chlorobium pheophytin prepared by Stanier & Smith (1960) by removing the magnesium from the chlorophyll of green bacteria.

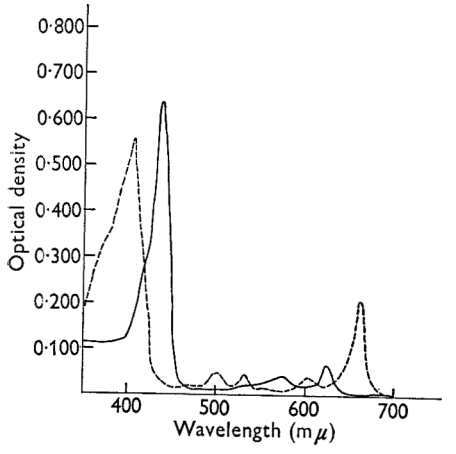


Fig. 3

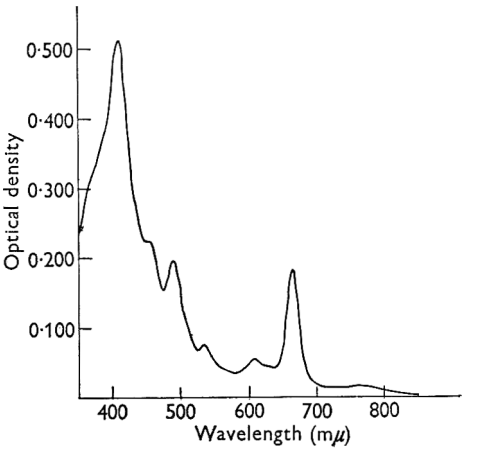


Fig. 4

Fig. 3. Absorption spectra of the pigments of the *Rhodopseudomonas spheroides* 660 mutants (---) and of the 625 mutants (—) after partial purification by chromatography. In ethyl ether.

Fig. 4. Absorption spectrum of an acetone+methanol extract of cells of a mutant of *Rhodopseudomonas spheroides*, lacking bacteriochlorophyll, which apparently grew under photosynthetic conditions.

Table 1. *Absorption maxima and relative absorption of the* 660 *mutants of* Rhodopseudomonas spheroides *compared with those of pheophytin* a (*Smith & Benitez*, 1955) *and of a Chlorobium pheophytin* (*Stanier & Smith*, 1960) *in ether*

Pheophytin *a*	max. mμ.	667	609·5	560	534	505	471	408·5
	rel. abs.	48·4	7·4	2·7	9·5	11·0	3·9	100
Chlorobium pheophytin (650)	max. mμ.	660	605	555	532	503	470	405
	rel. abs.	49·6	6·6	2·5	9·8	8·9	3·1	100
660 mutants	max. mμ	660	603	555	531	502	470	405
	rel. abs.	37·8	7·0	2·7	8·1	8·8	3·4	100

625 *mutants.* For this group of mutants the spectrum of the partially purified pigment shown in Fig. 3 has a long wavelength maximum at 625 mμ; a further displacement of 35 mμ to shorter wavelengths when compared with the 660 mutants. The highest peak was at 437 mμ, with minor peaks at 535 and 573 mμ. The position of the peak at 625 mμ indicates that the pigment was at a still higher oxidation level than that of the 660 mutants, and was at the same oxidation level as protochlorophyll. The relative heights of the smaller peaks show that, in contrast to the 660

mutants, the pigment of the 625 mutants did have magnesium in the molecule. Comparison with published spectral data (Koski & Smith, 1948) shows that the 625 pigment is very closely related to protochlorophyll (Table 2).

Table 2. *A comparison of the wavelengths and relative intensities of the absorption maxima of protochlorophyll (Koski & Smith,* 1948) *and the* 625 *mutants of* Rhodopseudomonas spheroides *in ether*

Protochlorophyll	max. mμ	623	571	535	432
	rel. intensity	2	3	4	1
625 mutants	max. mμ	624	573	535	437
	rel. intensity	2	3	4	1

Biological observations

Stability of mutants. Both groups of mutants were unstable in several respects. There was frequent loss by death on slopes for no obvious reason. In all the mutants studied there was a progressive loss of pigment with time, which made it impossible to conduct all the work on any one mutant. When put under photosynthetic conditions in agar stabs or liquid culture, all the mutants, with a single exception, initially reverted to the wild-type, some more readily than others. However, two mutants examined more than three years after their isolation were found to have lost completely their original capacity to revert to the wild-type.

Photosynthetic ability of mutants. No evidence of any photosynthetic ability in the 625 group of mutants was obtained, but work with this group was minimal. The results with the 660 groups of mutants were less clear-cut. The earliest mutant of this type was isolated in 1945 (UV 47); it gave rise to colonies of a pale brown-green colour. When first isolated, it was extremely unstable under photosynthetic conditions; subsequent plating produced colonies of a great variety of colours, from almost colourless, to dark red, with several green and tan, as well as the normal brown-green. In a stab it usually showed light growth along the length of the stab, rather than centres of reversion. This suggested limited photosynthetic ability, but plating of organisms from such a stab invariably showed some wild-type colonies, so the result was inconclusive.

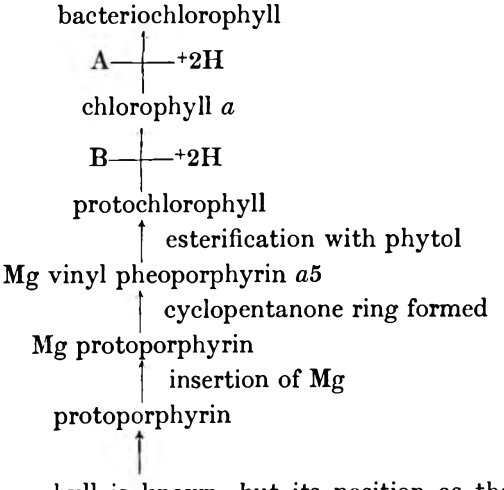
In another experiment mutant UV 47 was grown semi-aerobically in the dark, and while in the exponential phase of growth was transferred to closed tubes in the light. Forty-eight hours later the tubes showed a dense growth which was bright green: the organisms were green and the medium was green, whereas with the wild-type the organisms are red and the medium either colourless or pink under these conditions. The spectrum of an acetone + methanol extract of organisms showed that most of the pigment present was of the 660 mμ kind, with only traces of bacteriochlorophyll, absorbing at 760 mμ, due to a few reverted organisms (Fig. 4). The major photosynthetic growth had apparently been due to mutant type organisms, but the result was again inconclusive because of the presence of some reverted organisms. Further experiments, designed to obtain quantitative results, failed since the mutant was soon unable to grow at all under photosynthetic conditions. A similar transient ability to photosynthesize was found in two other mutants of the 660 groups.

DISCUSSION

During this and previous investigations, groups of mutants have been isolated which show changes in pigment synthesis. These fall into five main groups which are summarized in Table 3, which shows the nature of the pigments they contain and the photosynthetic ability of the members of each group. This does not exhaust the theoretical possibilities. No mutant has been found from which both carotenoid and their colourless precursor phytoene are absent, but in which bacteriochlorophyll is present. This may be an impossible combination from a mathematical point of view, as bacteriochlorophyll apparently occurs only in chromatophores, and carotenoids or their precursors appear to be necessary components of these structures. Nor has a group of mutants been found in which a bacteriochlorophyll precursor is present but carotenoids absent. This might be for the same reason, or perhaps two mutations are required for such a change. In this connexion the last group of mutants is of interest in that the loss of all pigments has apparently been brought about by a single mutation.

In the wild-type bacteriochlorophyll precursors have not been detected in the cells by the methods used. Blocked mutants, which presumably accumulate the precursor formed immediately before the block, are therefore useful in indicating biosynthetic pathways. It was hoped that the two new groups of mutants isolated might throw light on the biosynthetic pathway of bacteriochlorophyll.

Granick (1951) worked out a scheme, based on his work on the green alga Chlorella, for the major features of the synthesis of green plant chlorophyll. He suggested that bacteriochlorophyll fits into the scheme as follows:



The structure of bacteriochlorophyll is known, but its position as the terminal molecule in such a biosynthetic pathway is mainly speculative and based on chemical reasoning. The two immediate precursors of bacteriochlorophyll, according to this scheme, would be chlorophyll *a* and protochlorophyll, and these compounds might be expected to accumulate in mutants blocked at A and B respectively. The pigment of the 660 mutant of *Rhodopseudomonas spheroides* is clearly not chlorophyll *a* although it is a related compound at the same oxidation level as chlorophyll *a*. Likewise, the pigment of the 625 mutant of *R. spheroides* is not protochlorophyll,

although it is a very closely related compound at the same oxidation level. The difficulty in interpretation arises from the fact that although the 625 mutant pigment contains magnesium (as would be expected from Granick's scheme since it should be inserted relatively early in the biosynthesis of bacteriochlorophyll) the 660 pigment lacks magnesium and cannot be the precursor of bacteriochlorophyll. Perhaps in the 660 mutants the molecule occurring in the cell has, in fact, a magnesium atom in it, but is unstable during extraction; more subtle chemical extraction methods might reveal this. One can conclude only that pigments at oxidation levels consistent with Granick's scheme have been found. However, the fact that the 660 pigment resembles the pheophytin from Chlorobium chlorophyll even more closely than pheophytin from chlorophyll *a* suggests that the former may be the actual precursor of bacteriochlorophyll. In this case, the terminal stages in bacteriochlorophyll synthesis would proceed by steps parallel to, but not identical with, those for chlorophyll *a*.

Table 3. *The pigments contained in groups of mutants obtained by irradiation of* Rhodopseudomonas spheroides

Mutant group	Bacteriochlorophyll	Carotenoids	Photosynthetic ability
I	Normal	Present, but different from wild-type	Present
II	Normal	Absent, but colourless precursor phytoene, present	Present under anaerobic conditions
III	Absent, but 660 or 625 mμ dervative of bacteriochlorophyll present	Normal	None or doubtful
IV	Absent	Normal	None
V	Absent	Absent	None

Granick (1957) suggested that in the evolution of photosynthetic pigments the precursors of the current pigments were at one time the functional light absorbers for photosynthetic reactions. They ceased to have this role when they gave up their positions as terminal members on the biosynthetic pathway, but he suggested that certain vestigial capacities might remain. During the present work the question arose as to whether or not any of the 660 mutants of *Rhodopseudomonas spheroides* could photosynthesize. It was not at first realized that the extracted pigment of the 660 mutants contained no magnesium, or experiments to try to force photosynthetic growth would not have been attempted. It seems impossible to explain the dense growth of one 660 mutant (UV 47), anaerobically in the light, except on the basis of a transient capacity to photosynthesize. One possibility is that a magnesium containing pigment, closely related to chlorophyll *a*, is formed by at least some of the 660 mutants, but is unstable in the cell and accumulates for only a short time.

This work was supported by a grant from the National Science Foundation.

REFERENCES

COHEN-BAZIRE, G., SISTROM, W. R. & STANIER, R. Y. (1957). Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. cell. comp. Physiol.* **49**, 25.

GRANICK, S. (1951). Biosynthesis of chlorophyll and related pigments. *Annu. Rev. Pl. Physiol.* **2**, 115.

GRANICK, S. (1957). Speculations on the origins and evolution of photosynthesis. *Proc. nat. Acad. Sci., Wash.* **69**, 292.

GRIFFITHS, M. & STANIER, R. Y. (1956). Some mutational changes in the photosynthetic pigment system of *Rhodopseudomonas spheroides*. *J. gen. Microbiol.* **14**, 698.

KOSKI, V. M. & SMITH, J. H. C. (1948). The isolation and spectral absorption properties of protochlorophyll from etiolated barley seedlings. *J. Amer. chem. Soc.* **70**, 3558.

RABINOWITCH, E. I. (1951). *Photosynthesis and Related Processes*, vol. 2, part I. New York: Interscience Publishers.

SISTROM, W. R., GRIFFITHS, M. & STANIER, R. Y. (1956). A note on the porphyrins excreted by the blue-green mutant of *Rhodopseudomonas spheroides*. *J. cell. comp. Physiol.* **48**, 459.

SMITH, J. H. C. & BENITEZ, A. (1955). Chlorophylls: analysis in plant materials. *Moderne Methoden der Pflanzenanalyse*, **4**, 142. Berlin: Springer-Verlag.

STANIER, R. Y. & SMITH, J. H. C. (1960). The chlorophylls of green bacteria. *Biochim. biophys. Acta*, **41**, 478.

STERN, A. & WENDERLEIN, H. (1936). Über die Lichtabsorption der Porphyrine. *Z. phys. Chem.* A, **176**, 81.

STERN, A. & PRUCKNER, F. (1939). Lichtabsorption einiger Derivate des Bacteriochlorophylls. *Z. phys. Chem.* A, **185**, 140.

J. gen. Microbiol. (1962), **27**, 437–449
With 1 *plate*
Printed in Great Britain

Nutritional Control of Cellular Form in *Trigonopsis variabilis*

By S. SENTHESHANMUGANATHAN* and W. J. NICKERSON
Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey, U.S.A.

(*Received* 9 *May* 1961)

SUMMARY

The cellular form of *Trigonopsis variabilis* may be either triangular or ellipsoidal in outline. Development of form in this yeast is subject to nutritional control. Cell populations, almost exclusively of triangular form, are obtained in defined media containing methionine, or other suitable methyl group donor. Cultures in defined media devoid of methyl group donor usually consist exclusively of ellipsoidal forms.

INTRODUCTION

In studies on the biochemical bases of morphogenesis in micro-organisms which have been conducted in this laboratory, yeast ↔ filament transformation in *Candida albicans* has been investigated in detail (Nickerson, 1954; Falcone & Nickerson, 1959), and filament ↔ yeast conversion in *Mucor rouxii* has been explored (Bartnicki-Garcia & Nickerson, 1959). More recently, an intriguing 'triangular' yeast (Trigonopsis) and 'lemon-shaped' yeasts (Kloeckera) have excited our curiosity. We have inquired into molecular bases of form development in the ellipsoidal ↔ triangular transformation of Trigonopsis, and present our findings on experimental control of this transformation. To designate a remarkable 'triangular' yeast Schachner (1929) proposed the generic name *Trigonopsis*; to stress his observation that cell populations of this organism consisted of both triangular and ellipsoidal forms, he selected *variabilis* as the specific designation. The unusual morphology of this yeast poses an intriguing problem in respect to its morphogenesis. The cellular envelope determines cellular form in a yeast; what differences in wall composition could result in abrupt changes such that an ellipsoidal form could develop a triangular bud? What arrangement of polymeric components in a wall fabric would form a triangular lozenge? Early in the course of the present work it was found that development of cellular form in *Trigonopsis variabilis* is subject to nutritional control. Nutrients that constitute donors of active methyl groups, and thus promote phospholipid synthesis, were found to foster the development of populations consisting exclusively of triangular forms. Conversely, cultures in defined media devoid of methyl donors were observed to consist exclusively of ellipsoidal forms of lower phospholipid content. To account for the molecular basis of form in *T. variabilis*, the composition and structure of isolated cell walls was

* Present address: Medical Research Institute, Colombo 8, Ceylon.

examined. Architectural and physical properties of polymeric components of the wall fabric were examined because of their role as ultimate determinants of cellular form. The present paper is devoted to the nutritional control of morphogenesis in *T. variabilis*.

METHODS

Microbiological methods. The organism used in this work was *Trigonopsis variabilis* Schachner (strain CBS 1040) obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, Holland. Cultures were grown on a defined medium which contained (all quantities/100 ml.): glucose, 2 g.; KH_2PO_4, 0·4 g.; $MgSO_4.7H_2O$, 0·1 g.; $(NH_4)_2SO_4$, 0·2 g.; $CaCl_2$, 0·05 g.; H_3BO_3, 10 mg.; ammonium molybdate, 4 mg.; $MnSO_4.4H_2O$, 4 mg.; $ZnSO_4.7H_2O$, 4 mg.; $CuSO_4.5H_2O$, 4·5 mg.; $FeSO_4.7H_2O$, 2·5 mg. In preliminary studies, before the vitamin requirements of the yeast were ascertained, a supplement consisting of the following nutrilites was included: 400 μg. each of calcium (+) pantothenate, nicotinic acid, pyridoxine.HCl and thiamine.HCl; 200 μg. each of folic acid, inositol, riboflavin and *p*-aminobenzoic acid; 2 μg. of biotin. The medium was adjusted to pH 6·0, and sterilized by autoclaving at 115° for 20 min. Glucose was sterilized separately by autoclaving. For studies of vitamin requirements, nutrilite solutions were sterilized by filtration.

Cultures were grown in conical flasks of a capacity two and a half times the volume of medium used. The inoculum was 0·1 ml. of a washed suspension of organism (optical density 0·200, as determined in a Klett-Summerson photo-electric colorimeter with filter No. 54) obtained from a 72 hr. liquid culture that had been grown in the above medium. After inoculation, cultures were incubated at 28° for 70–72 hr. on a rotatory shaker which was set at 250 rev./min. Stock cultures were maintained on slopes of the above medium solidified with 2 % (w/v) agar (Difco). When the vitamin requirement studies were completed, the standard medium was modified to include only two vitamins, namely, biotin (2 μg./100 ml.) and thiamine HCl (10 μg./100 ml.). After ascertaining the influence of nitrogen source on development of cellular form, the 'basal medium' was defined to contain: glucose, salts, biotin and thiamine.

Measurement of growth. Unless stated to the contrary, all growth measurements were made at the end of incubation for 72 hr. at 28°, and are expressed in terms of mg. dry weight organism per litre. Dry weight was determined by reference to a standard graph relating optical density to dry weight. Optical density was determined turbidimetrically with a Klett-Summerson photo-electric colorimeter, with filter No. 54, and uninoculated medium as reference.

Counts of organisms. Dry smears of cultures were made and stained negatively with nigrosin (0·1 %, w/v) to reveal distinctly the outline of the organisms; counts were made of at least 250 organisms.

Reagents. The purity of amino acids, obtained from commercial sources, was ascertained by paper chromatography (Woiwood, 1949). The isomeric forms of compounds used are indicated in the text. DL-Methionine was purchased from Sigma Chemical Co., St Louis, and was recrystallized three times from hot water until chromatographically pure. D- and L-methionine, DL-homocysteine and DL-homocystine were obtained from Mann Research Laboratories, New York, as chromatographically pure preparations. Ethionine and choline were obtained from

Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.; inositol from Corn Products Co., 17 Battery Place, New York; and glyceromono-oleate from Glyco Products Co., Inc., Brooklyn 2, New York. This latter compound was free from any trace of biotin when estimated by the chromatographic method of Nickerson (1961) with *Candida albicans* 582 as test organism. Oleic acid was obtained from the California Corporation for Biochemical Research, Los Angeles, California. Lecithin (soya bean; Nutritional Biochemicals Corp., Cleveland, Ohio) was purified by treatment with acetone. The pyrimidine (4-amino-2-methyl-pyrimidyl-5-methyl-sulphonic acid) and thiazole (4-methyl-5-β-hydroxyl thiazole) moieties of thiamine were prepared from thiamine by the method of Williams, Waterman, Keresztesy & Buchman (1935). Preparations thus obtained were free from thiamine when tested by the thiochrome method (Hennessy & Cerecedo, 1939). Glucose oxidase (Glucostat) was obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

Solutions. Solutions of glyceromono-oleate and oleic acid were prepared in ethanol, sterilized by filtration and added aseptically to culture medium. Homocystine and homocysteine were dissolved in water immediately before use and sterilized by filtration.

Purification of agar. Difco Bacto agar (50 g.) was suspended in 500 ml. distilled water, stirred for 15 min. and filtered; this treatment was effected twice. The washed agar was then shaken in 200 ml. of 95 % (v/v) ethanol in water and filtered. The residue was resuspended in 500 ml. distilled water, filtered, and washed again with 200 ml. of 95 % (v/v) ethanol in water. Material thus obtained was allowed to dry overnight. The agar was scraped from the filter and stirred with aqueous pyridine (100 ml. redistilled pyridine + 350 ml. water) for 15 min. and filtered; this process was repeated twice. Finally, the material was washed with ethanol, then acetone and dried. The material thus obtained was free from thiamine and biotin; *Trigonopsis variabilis* did not grow when these two vitamins were omitted from growth medium solidified with this washed agar.

Preparation of silica gel plates. To defined medium containing either $(NH_4)_2SO_4$ or DL-methionine as nitrogen source, sufficient silica gel was added to give a final concentration of 5 % (w/v), and adjusted to pH 5·9 with dilute NaOH. This mixture was poured into plates and autoclaved at 120° for 20 min. (see Falcone & Nickerson, 1958). Plates were seeded with a washed suspension of organisms which had been grown in $(NH_4)_2SO_4$ basal medium containing thiamine (10 μg./100 ml.). Plates were incubated at 28° in a water-saturated atmosphere to prevent rapid drying of the medium.

RESULTS

Nutritional requirements of Trigonopsis variabilis

Growth with different carbohydrate sources. Carbohydrate requirements of *Trigonopsis variabilis* were examined by Lodder & Kreger-van Rij (1952) in their taxonomic study of yeasts; they reported that this organism grew only with glucose or galactose as sources of carbohydrate. Our results (Table 1) are in agreement, except that we also observed moderate growth with sucrose; the sucrose used was ascertained to be free from glucose, as judged by the glucose oxidase method. After incubation for 72 hr., growth with glucose, fructose or mannose was in the ratio 1·6:1·6:1·0. The effect of glucose concentration on the rate of growth was also

examined. Neither rate of growth nor amount of growth after 72 hr. changed appreciably above a glucose concentration of 2 % (w/v).

Growth in presence of different amino acids. There are no available data about the ability of this yeast to utilize any nitrogen source other than $(NH_4)_2SO_4$. Of thirteen amino acids tested as sole source of nitrogen (Table 2), eleven supported growth (DL-isoleucine and L-valine were not suitable as sole sources of nitrogen). Growth was most marked with alanine or proline as nitrogen source (900 mg. dry wt./l.); asparagine and leucine gave an intermediate response, whilst with

Table 1. *Growth of* Trigonopsis variabilis *with different carbohydrate sources*

Medium, with vitamin supplement, as described in text; carbohydrate concentration, 2 % (w/v); total volume, 50 ml. The values (mg. dry wt./l.) are the averages of triplicate cultures from two sets of experiments.

	Incubation period (hr.)				
	24	44	56	68	72
Carbohydrate	Growth (mg. dry wt. organism/l. culture)				
None	0	0	0	0	0
Glucose	4	126	514	636	655
Galactose	4	84	276	536	580
Sucrose	4	44	84	88	88
Maltose	2	2	2	3	3
Lactose	2	2	2	3	2

Table 2. *Growth of* Trigonopsis variabilis *with different amino acids as sources of nitrogen*

Conditions as described in Table 1. The nitrogen sources were supplied at a level of 200 mg. L- or 400 mg. DL-form per 100 ml. The values (mg. dry wt./l.) are the averages of duplicate cultures from two sets of experiments.

	Incubation period (hr.)		
	42	72	Shape of cells after 72 hr. incubation†
Nitrogen source	Growth (mg. dry wt./l.)		
None	0	0	—
$(NH_4)_2SO_4$	95	700	e
L-aspartic acid	24	128	e
L-asparagine	40	648	e
DL-α-alanine	210	970	△ and e
L-cysteine	24	58	e
Na-glutamate	14	116	e
L-histidine	8	260	e
L-leucine	48	695	e
DL-isoleucine	2	10	e
DL-methionine	16	268	△
L-proline	210	920	△ and e
DL-phenylalanine	8	90	e
DL-threonine	6	75	e
$(NH_4)_2SO_4$ + DL-alanine*	150	920	e
L-valine	10	30	e

* 100 mg. $(NH_4)_2SO_4$ + 100 mg. DL-alanine/100 ml.
† e = ellipsoidal; △ = triangular.

aspartate, glutamate, histidine or methionine, growth was less than one-seventh of that obtained with alanine or $(NH_4)_2SO_4$.

Morphology of populations in amino acid media. Cellular morphology was observed to vary with the nitrogen source supplied. With alanine and proline, the population was a mixture of triangular and ellipsoidal forms, whilst with ammonium sulphate and the remaining amino acids (except methionine) more than 97 % of the organisms were ellipsoidal. In contrast, more than 97 % of the organisms

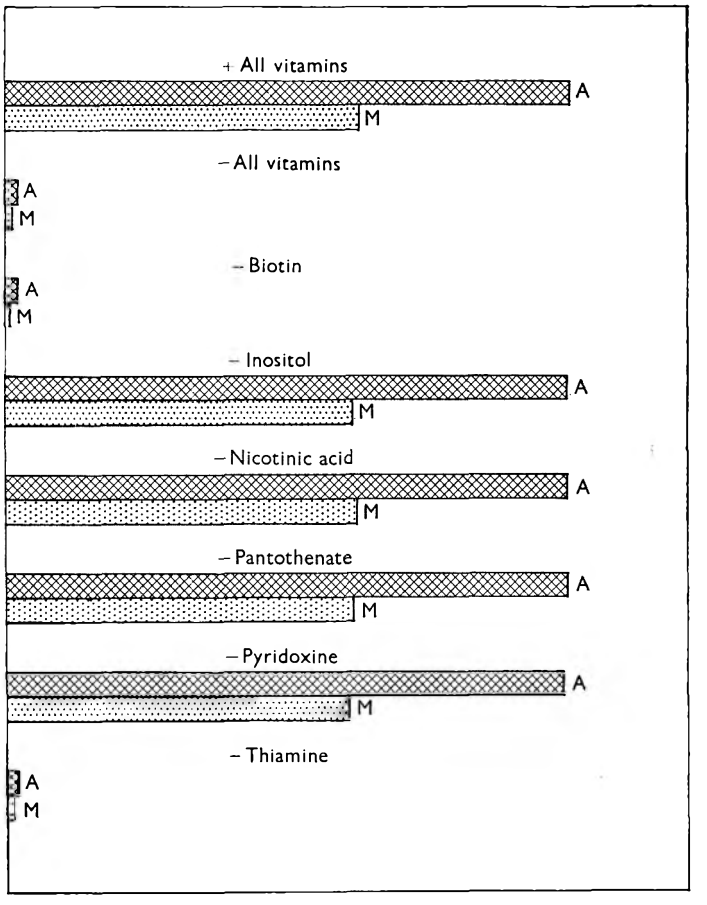


Fig. 1. Vitamin requirements of *Trigonopsis variabilis* with $(NH_4)_2SO_4$ or DL-methionine as sole source of nitrogen. Basal medium and growth conditions as described in text. Incubation period: 72 hr. A = $(NH_4)_2SO_4$ as sole source of nitrogen; M = DL-methionine as sole source of nitrogen.

were of triangular outline when methionine was the nitrogen source (see Table 2). It should be emphasized that organisms in cultures incubated for less than 48 hr. were all ellipsoidal, irrespective of the nitrogen source.

Vitamin requirements. Growth factor requirements of *Trigonopsis variabilis* were studied with methionine or $(NH_4)_2SO_4$ as nitrogen sources. In Fig. 1, it is shown that this yeast required only biotin and thiamine for optimal growth. Addition of

other vitamins, either singly or in mixtures, did not produce further increase in rate or amount of growth. The potential usefulness of this yeast for the assay of thiamine was explored. The response to thiamine was linear over a range from about 0 to 7 μg./100 ml. (Fig. 2), despite the fact that growth with ammonium sulphate was almost double that with methionine as source of nitrogen. This finding serves to emphasize that the source of nitrogen cannot be neglected in the design of basal media for vitamin assays.

When supplied with the thiazole moiety of thiamine, *Trigonopsis variabilis* grew substantially as well as when supplied with pre-formed thiamine (Table 3). Since

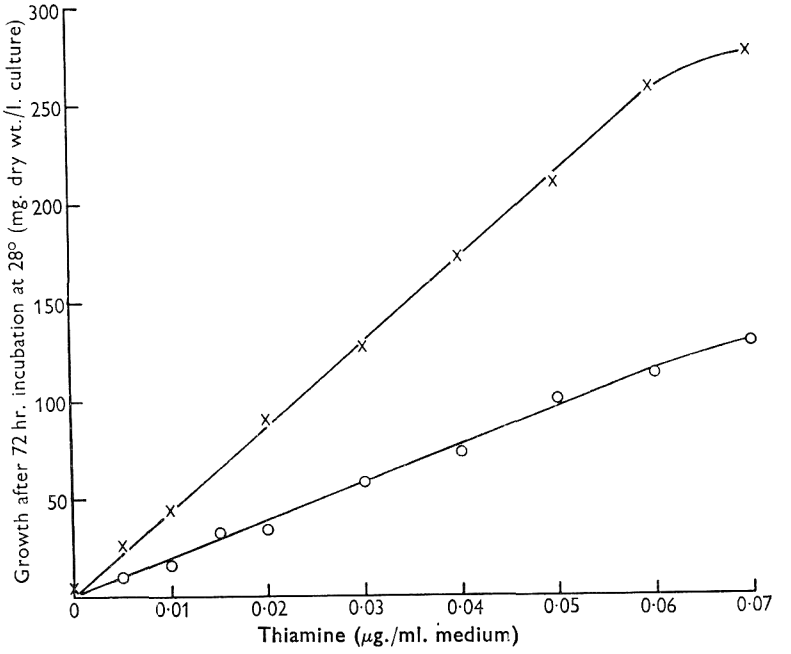


Fig. 2. Effect of thiamine concentration on the growth of *Trigonopsis variabilis*. ×—× $(NH_4)_2SO_2$ as N source (25 mg. N/100 ml.); ○—○ DL-methionine as N source (25 mg. N/100 ml.).

Table 3. *Effect of pyrimidine and thiazole moieties of thiamine on the growth of* Trigonopsis variabilis

Medium contained biotin (2 μg./100 ml.) in addition to thiamine moiety. The nitrogen source was supplied at 25 mg. N/100 ml. The growth factors (1 μg./ml.) were sterilized by filtration and added to the medium aseptically. The values for amount of growth (mg. dry wt./l.) are the averages of duplicate cultures from two sets of experiments after incubation for 72 hr. at 28°.

Growth factor	$(NH_4)_2SO_4$ medium	DL-methionine medium
	growth (mg. dry wt./l.)	
None	0	0
Thiamine	760	480
Pyrimidine	6	6
Thiazole	700	400
Thiamine + pyrimidine	700	400
Thiazole + pyrimidine	700	400

the pyrimidine moiety used contained a sulphonic acid group, the possibility of its acting as an inhibitory agent was considered. However, mixtures of the pyrimidine, with thiamine and with the thiazole moiety, gave no evidence of such action. It may be concluded, therefore, that biotin and the thiazole moiety of thiamine are essential nutrient factors for *T. variabilis*.

Partial replacement of the biotin requirement. It was shown by Nickerson (1961) for a baker's yeast strain and for *Candida albicans* 582 (an organism which requires only biotin as a growth factor), that biotin was absolutely replaceable by glycero-mono-oleate (GMO) at 30 mg./l. ($8{\cdot}5 \times 10^{-5}$ M). Oleic acid was without such effect for these yeasts. Above 40 mg./l., GMO was toxic for both of these organisms. It was found that *Trigonopsis variabilis* could also utilize GMO to replace biotin as a growth factor. With GMO growth occurred to an extent of about 25 % of that with biotin, and a pronounced lag was evident. When GMO was incorporated in the medium with biotin, a stimulation in the rate of growth was observed. At concentrations above 40 mg./l., GMO suppressed growth.

Nutritional control of morphogenesis in Trigonopsis variabilis

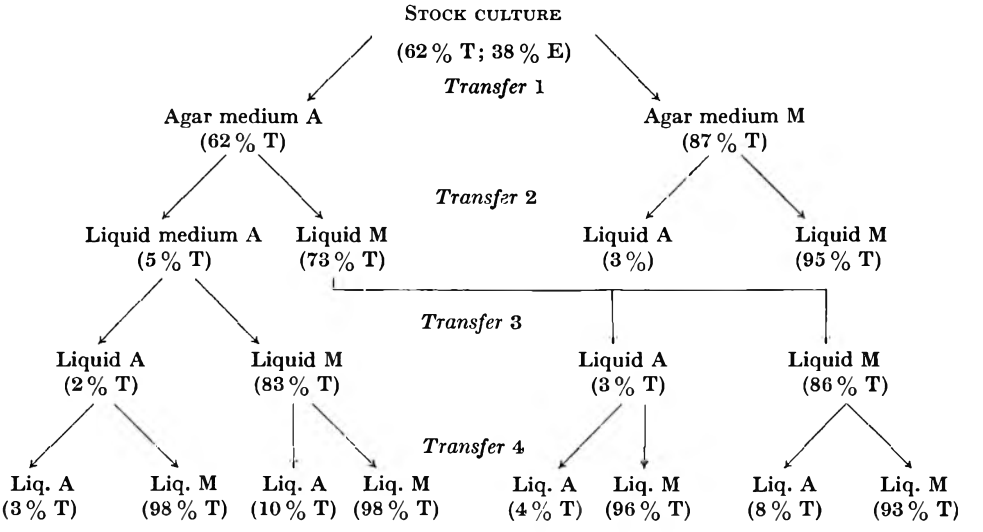
Growth with different isomers of methionine. In view of significant differences in form between organisms grown with methionine or with ammonium sulphate as sole source of nitrogen, further investigations with these nitrogen sources were made. As shown in Fig. 3, rates of growth of *Trigonopsis variabilis* with D- or DL-methionine were identical, and greater than that with L-methionine. The D- and L-amino acids used for this study were tested for contamination with the other isomer by the use of D-amino acid oxidase (prepared from hog kidney cortex according to Meister, 1952). The isomers were thus found to be of greater than 99 % purity. These observations suggest that under aerobic conditions D-methionine is deaminated at a faster rate than the L-isomer, implying that different enzyme systems are involved. These, and related results obtained with cell-free systems, will be described in a subsequent paper.

Form of growth of Trigonopsis variabilis *in liquid cultures.* On transfer from stock culture (yeast extract + glucose agar) to medium M (basal medium + DL-methionine), 87 % triangular and 13 % ellipsoidal forms were obtained after incubation for 3 days (Table 4). Transfer from solid medium M to liquid medium A resulted in 3 % triangular and 97 % ellipsoidal, whereas transfer to liquid medium M yielded 95 % triangular and 5 % ellipsoidal. It is reasonably clear that development of triangular forms on methionine medium is attributable to nutritional control of form-development and not to selection from a mixed population. Repeated transfer from liquid medium M to A, or transfer to medium A after many passages in liquid medium M always resulted in the prompt development of an exclusively ellipsoidal population. Conversely, transfer to liquid medium M after many passages in medium A always resulted in the development of about 95 % triangular forms after a culture had been incubated for 72 hr. In view of the incubation time in this case, a question as to the operation of a population-selection phenomenon naturally arises. As shown in Fig. 3, however, *T. variabilis* enters the logarithmic phase of growth in methionine liquid medium only after about 40 hr. of incubation. This result was obtained even on frequent and repeated passage in medium M. In medium A, the logarithmic phase of growth begins after about 28 hr. of incubation. With either medium the

logarithmic phase persists until about the 50th hr. After about 48 hr. of incubation in medium M appreciable numbers of triangular forms are evident (40 % of the total growth has been achieved by this time), and after about 70 hr. of incubation about 95 % of all organisms are triangular forms. Conversion of ellipsoidal forms to triangular forms occurs in the stationary phase; buds formed toward the end of the logarithmic phase are predominantly triangular. Repeated and frequent passage in

Table 4. *Form of* Trigonopsis variabilis *on repeated transfer from solid to liquid medium containing either methionine (M) or* $(NH_4)_2SO_4$ *(A) as sole source of nitrogen*

Standard basal medium was used; agar medium solidified with vitamin-free agar (2 %, w/v). Nitrogen source added at 25 mg. N/100 ml. The percentage of triangular (T) and ellipsoidal (E) forms was calculated from a total count of 250 organisms.



medium M does not alter the sequence of events just depicted. An inoculum comprised almost exclusively of triangular forms gives rise after a pronounced lag to ellipsoidal forms which, in turn, are converted into or give rise to triangular forms with onset of the stationary phase. Triangular organisms formed buds at the apices of the triangle; the budded young cells might be triangular or ellipsoidal. In many instances, ellipsoidal forms were found to bud and to give rise to triangular forms. The different forms of these organisms can be observed in the photomicrographs (Fig. 4).

Cultures on solidified media. With agar medium, irrespective of nitrogen source, the results were not consistent; sometimes as few as 75 % triangular forms were obtained in medium M. On the other hand, the results obtained with liquid media were very uniform and variations were only about 5–7 %. Variability in results obtained with cultures on solid medium A or M might result from the presence of extraneous material in the agar, but experiments with purified agar yielded similarly variable results. Experiments were then made in shake cultures containing 0·3 % (w/v) agar (Table 5). When agar was added to liquid medium A, the proportion of triangular forms increased sharply from 5 to 66 %, whereas the addition of agar to medium M resulted in a slight decrease in the proportion of triangular forms. The

experiments just outlined were repeated in media solidified with silica gel, but not more than 88 % triangular forms was obtained in medium M, whereas the proportion of triangular forms in medium A was of the order of 65 % (Table 5). We have no explanation for this discrepancy between the results obtained with liquid and those on solidified or semi-solid media.

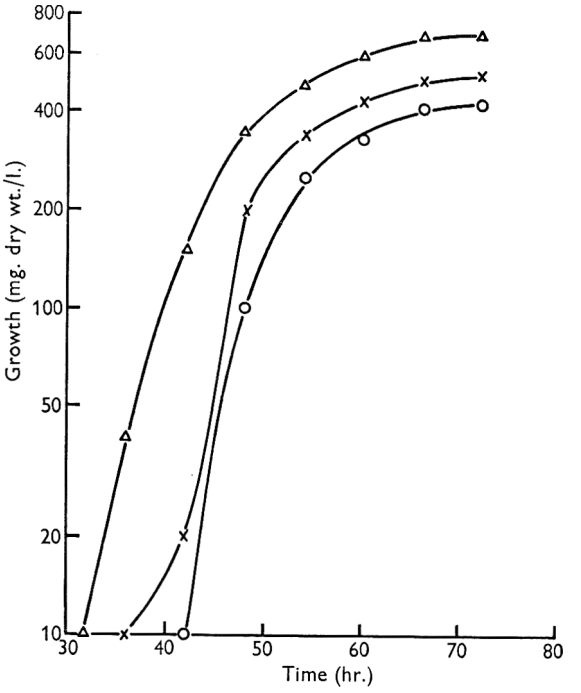


Fig. 3. Growth of *Trigonopsis variabilis* with D-, L-, or DL-methionine or $(NH_4)_2SO_4$ as sole source of nitrogen (25 mg. N/100 ml.). Basal medium, as described in text, supplemented with biotin (2 μg./100 ml.) and thiamine (10 μg./100 ml.). △·····△ $(NH_4)_2SO_4$; ×·····× D- or DL-methionine; ○·····○ L-methionine.

Table 5. *Effect of solidifying agents on the morphology of* Trigonopsis variabilis

To the basal medium, agar was added (where indicated) to a final concentration of 0·3 % (w/v). Silica gel plates were prepared as described in text. Inocula consisted of organisms grown in liquid medium with $(NH_4)_2SO_4$ as nitrogen source. All experiments were performed twice, in duplicate.

Additions to basal medium	Form of organisms (%)	
	Triangular	Ellipsoidal
Expt. 1 (semi-solid agar)		
$(NH_4)_2SO_4$	5	95
$(NH_4)_2SO_4$ + agar	66	34
DL-Methionine	90	10
DL-Methionine + agar	75	25
Expt. 2 (silica gel)		
$(NH_4)_2SO_4$ (10 mg.)	65	35
$(NH_4)_2SO_4$ (25 mg.)	68	32
DL-Methionine (10 mg.)	88	12
DL-Methionine (25 mg.)	82	18

Effect of transmethylating agents on morphology. Several compounds, such as methionine, choline, betaine or dimethyl-β-propiothetin can serve as methyl donors. Stekol *et al.* (1953) reported that synthesis of choline (a component of phospholipids) from dimethylaminoethanol involves a transmethylation reaction in which methionine supplies a preformed methyl group. Thus, in promoting morphological change in *Trigonopsis variabilis*, methionine might supply preformed methyl groups for the synthesis of choline or choline-containing compounds such as lecithin. If so, the use of homocysteine (the 'demethylated' precursor of methionine) or homocystine, as sole source of nitrogen, should result in predominantly ellipsoidal populations.

Table 6. *Effect of choline, inositol and folic acid on growth and form of* Trigonopsis variabilis *with precursors or antagonist of methionine*

Additions to basal medium	Growth (mg. dry wt./l.)	Form of organism (%)	
		Triangular	Ellipsoidal
Expt. 1 (*Homocysteine*)			
$(NH_4)_2SO_4$	782	10	90
DL-Methionine	350	93	7
DL-Homocysteine (HCSH)	113	15	85
HCSH + choline (cho)	158	16	84
HCSH + inositol (inos)	91	20	80
HCSH + folic acid (fol)	105	8	92
HCSH + cho + inos	161	52	48
HCSH + cho + fol	149	68	32
HCSH + cho + fol + inos	180	88	12
Expt. 2 (*Homocystine*)			
$(NH_4)_2SO_4$	750	9	91
DL-Methionine	325	94	6
DL-Homocystine (HCS—SCH)	62	5	95
HCS—SCH + cho	245	67	33
HCS—SCH + inos	64	6	94
HCS—SCH + fol	110	6	94
HCS—SCH + cho + inos	240	60	40
HCS—SCH + cho + fol	265	71	29
HSC—SCH + cho + fol + inos	300	90	10
Expt. 3 (*Ethionine*)			
$(NH_4)_2SO_4$	648	8	92
DL-Methionine	321	92	8
Ethionine	34	—	—
Ethionine + DL-methionine	267	67	33
Ethionine + inos + cho	105	52	48

With homocysteine as nitrogen source (Table 6) there developed 85 % ellipsoidal forms and only 15 % triangular forms. The addition of choline did not alter this result, but when folic acid was also added, the proportion of triangular forms increased to about 65 %. Although the addition of inositol did not produce any further increase in the relative proportion of triangular forms, the mixture of folic acid, choline and inositol with homocysteine prompted the formation of triangular forms to an extent almost equal to that obtained with methionine alone. The results with homocystine as nitrogen source were similar to those just detailed, except that a noticeable increase in the proportion of triangular forms was obtained on supplementation with choline (67 % as compared to 5 % triangular when choline was omitted). Choline also stimulated growth in the presence of homocystine.

Ethionine (an antagonist for methionine) was not effective in supporting growth when supplied as sole source of nitrogen (Table 6, Expt. 3). When ethionine and methionine were added in equal weights, not only was growth inhibited by 20 % but the 'methionine effect' on the morphology was inhibited by about 25 %.

Effect of phospholipid moieties on morphology. The addition of inositol to medium A stimulated development of triangular forms; this may be taken as further evidence that form-development in *Trigonopsis variabilis* is dependent upon synthesis of lipids and phosphatides. To examine this hypothesis, experiments were conducted with components of phospholipids, e.g. choline, glycerol and glycerol esters. The results (Table 7) show that the addition of choline and glycerol to a medium containing $(NH_4)_2SO_4$ as nitrogen source increased the proportion of triangular forms (74 % as compared to 10 %), and glyceromono-oleate supported 22 % triangular forms. Although lecithin was found to inhibit growth, the two forms were found in equal numbers. The free and bound lipid contents of whole organisms and isolated walls of triangular forms were found to be substantially higher than in ellipsoidal forms (SentheShanmuganathan & Nickerson, 1962*b*).

Table 7. *Effect of phospholipid components on the growth and form of* Trigonopsis variabilis

To the standard basal medium containing $(NH_4)_2SO_4$ as sole source of nitrogen, the following, where noted, were added (per 100 ml.): choline, 20 mg.; glycerol, 9·2 mg.; glyceromono-oleate (GMO), 3·2 mg.; lecithin, 2 mg. The inoculum consisted of washed suspension of organisms grown with $(NH_4)_2SO_4$ as nitrogen source.

Additions to basal medium	Growth (mg. dry wt./l.)	Form of organism (%)	
		Triangular	Ellipsoidal
None	453	10	90
Choline	445	33	67
Choline + glycerol	540	74	26
GMO	510	22	78
Choline + lecithin	177	50	50

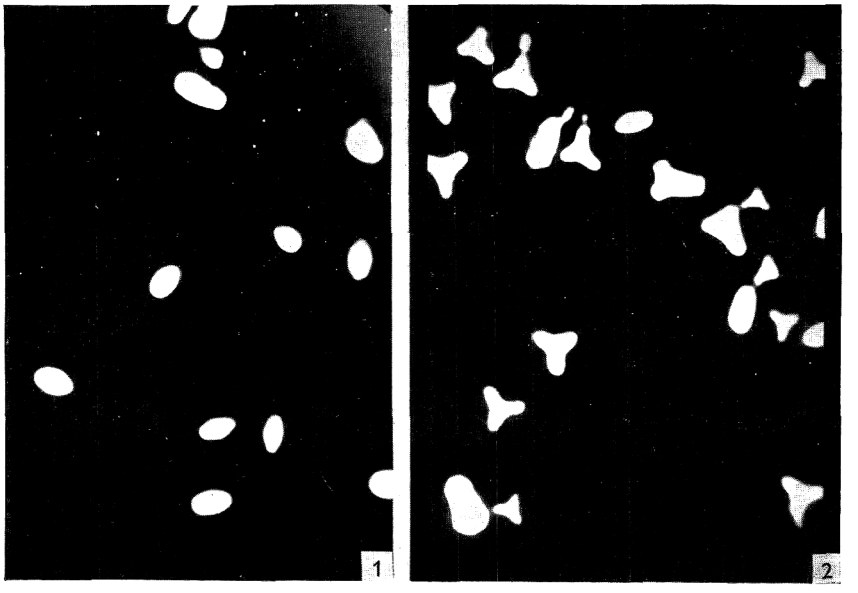
DISCUSSION

During studies on nutritional requirements of *Trigonopsis variabilis* it was observed that organisms obtained with methionine as sole source of nitrogen were mainly triangular, whereas those obtained with $(NH_4)_2SO_4$ were mainly ellipsoidal. This phenomenon of dimorphism was very marked in liquid cultures. An attempt was made to elucidate the manner in which nutritional control of form-development in this yeast is mediated biochemically. In addition to its appearance in most proteins, methionine (an indispensable component of the diet of higher animals) has been implicated in a wide variety of biosynthetic reactions: formation of lignin, nicotine, spermidine, creatine, adrenalin, anserine and cysteine (see Fruton & Simmons, 1958). All but the latter of these involvements are improbable in the present connexion. Methionine is known to be a member of the 'aspartate family' of amino acids in *Escherichia coli* (Roberts & Abelson, 1953) and in *Neurospora crassa* (Abelson & Vogel, 1955). Furthermore, Rose & Wixom (1955) have shown that a large part of the dietary requirement of man for methionine may be met by

feeding cystine (cysteine). Since other members of the aspartate family, as well as cysteine (cystine), are devoid of ability to stimulate the production of triangular forms, we may dismiss the many involvements growing out of these familial associations in our search for the *modus operandi* of methionine on morphogenesis of *T. variabilis*. Studies with homocysteine (homocystine), the methyl-free precursor of methionine, provided indications that the morphogenetic action of methionine resides in its operation as a methyl donor. Methionine was found to be replaceable either by choline + inositol (with ammonium sulphate as the nitrogen source) or by choline alone (with homocysteine as nitrogen source). These, and other data from nutritional studies, point strongly to an inter-relationship between phospholipid synthesis and the morphology of this yeast.

The two forms of *Trigonopsis variabilis* have been termed either 'triangular' or 'ellipsoidal' in outline. Naturally, a question arises as to the three-dimensional aspect of the 'triangular' forms. As shown in Pl. 1, fig. 2 (and especially, as shown in Fig. 1 of the following paper (SentheShanmuganathan & Nickerson, 1962*b*), this form is triangular in plane view and lozenge-shaped in cross-section; it is not tetrahedral nor pyramidal in three-dimensional aspect. The geometrical implications of transformation from an ellipsoidal pattern (prolate spheroid in three dimensions) to a triangular lozenge (three-dimensional aspect) resolve to those concerned with continuous deformation of a prolate spheroid which, without break in surface continuity, i.e. continuously differentiable deformation, lead to a triangular lozenge shape. In this transition, obviously, the surface/volume ratio is increased; simultaneously, surface tension at the organism/medium interface is decreased dramatically. Since measurements of gas transport (see SentheShanmuganathan & Nickerson, 1962*c*) are only modestly in favour of the triangular form, we are inclined to attach only secondary significance to alteration in surface/volume ratios. Moreover, in view of the nature of biosyntheses favoured by a methyl-donor environment and the effect thereof on cellular lipid-composition (SentheShanmuganathan & Nickerson, to be published), we feel that considerable importance must be attached to synthesis of polymeric components tending to minimize surface tension at the cell/medium interface. We may here be dealing with a biological example of the classical problem of Plateau (see Radó, 1951): 'to determine the surface of least area with a given boundary' (Bell, 1940). Such problems are easily visualized (as Plateau demonstrated) as soap films which span wire models of the boundary. Here, however, we are dealing with an underlying architecture that determines boundary shape and, hence, the surface of minimal area which encompasses the boundary. We shall defer further consideration of molecular bases of form development in this organism to the third paper in this series, where we shall return to a consideration of factors that determine the triangular boundary of this organism.

Supported in part by grants from the National Institutes of Health, U.S. Public Health Service. One of us (S.S.) was Pfizer Postdoctoral Fellow in Microbiology, on leave of absence from the Medical Research Institute, Colombo 8, Ceylon, when this work was done.


1
2

REFERENCES

ABELSON, P. H. & VOGEL, H. J. (1955). Amino acid biosynthesis in *Torulopsis utilis* and *Neurospora crassa*. *J. biol. Chem.* **213**, 355.

BARTNICKI-GARCIA, S. & NICKERSON, W. J. (1959). The yeast-like form of *Mucor rouxii*. *Proc. 9th Int. Bot. Cong., Montreal*, p. 22.

BELL, E. T. (1940). *The Development of Mathematics*, p. 354. New York: McGraw-Hill.

FALCONE, G. & NICKERSON, W. J. (1958). Physiological bases of morphogenesis in fungi. 6. Effect of sulfur-deficiency on growth and cellular division of *Candida albicans*. *G. Microbiol.* **4**, 105.

FALCONE, G. & NICKERSON, W. J. (1959). Enzymatic reactions involved in cellular division of microorganisms, in vol. 6, *Biochemistry of Morphogenesis*. *Proc. 4th int. Cong. Biochem., Vienna*, p. 65. London: Pergamon Press.

FRUTON, J. S. & SIMMONDS, S. (1958). *General Biochemistry*, 2nd ed. New York: John Wiley and Sons, Inc.

HENNESSY, D. J. & CERECEDO, L. R. (1939). The determination of free and phosphorylated thiamine by a modified thiochrome assay. *J. Amer. chem. Soc.* **61**, 179.

LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts, A Taxonomic Study*. Amsterdam: North Holland Pub. Co.

MEISTER, A. (1952). Enzymatic preparation of α-keto acids. *J. biol. Chem.* **197**, 309.

NICKERSON, W. J. (1954). An enzymatic locus participating in cellular division of a yeast. *J. gen. Physiol.* **37**, 483.

NICKERSON, W. J. (1961). Role of biotin in the multiplication of yeasts. *Bact. Proc.* p. 177.

RADÓ, T. (1951). *On the Problem of Plateau*. New York: Chelsea Publishing Co.

ROBERTS, R. B. & ABELSON, P. H. (1953). The role of the tricarboxylic acid cycle in amino acid synthesis in *Escherichia coli*. *Science*, **117**, 471.

ROSE, W. O. & WIXOM, R. L. (1955). The amino acid requirements of man. XIII. The sparing effect of cystine on the methionine requirement. *J. biol. Chem.* **216**, 763.

SCHACHNER, J. (1929). *Trigonopsis variabilis* nov.gen. et spec. *Z. ges. Brauw.* **52**, 137.

SENTHESHANMUGANATHAN, S. & NICKERSON, W. J. (1962*b*). Composition of cells and cell walls of triangular and ellipsoidal forms of *Trigonopsis variabilis*. *J. gen. Microbiol.* **27**, 451.

SENTHESHANMUGANATHAN, S. & NICKERSON, W. J. (1962*c*). Transaminase and D-amino acid oxidase of *Trigonopsis variabilis*. *J. gen. Microbiol.* **27**, 465.

STEKOL, J. A., WEISS, S., SMITH, P. & WEISS, K. (1953). The synthesis of choline and creatine in rats under various dietary conditions. *J. biol. Chem.* **201**, 299.

WILLIAMS, R. R., WATERMAN, R. E., KERESZTESY, J. C. & BUCHMAN, E. R. (1935). Preparation of pyrimidine and thiazole moieties of thiamin. *J. Amer. chem. Soc.* **57**, 536.

WOIWOOD, A. J. (1949). A method for the estimation of micro amounts of amino nitrogen and its application to paper partition chromatography. *Biochem. J.* **45**, 412.

EXPLANATION OF PLATE

PLATE 1

Photomicrographs of *Trignonopsis variabilis*. Outline shown in relief against nigrosin. Both types from liquid cultures incubated for 72 hr. at 28°.

Fig. 1. Ellipsoidal organisms grown in medium A, with ammonium sulphate as nitrogen source.

Fig. 2. Triangular forms grown in medium M with DL-methionine as nitrogen source.

J. gen. Microbiol. (1962), 27, 451–464
With 1 plate
Printed in Great Britain

Composition of Cells and Cell Walls of Triangular and Ellipsoidal Forms of *Trigonopsis variabilis*

By S. SENTHESHANMUGANATHAN* and W. J. NICKERSON
Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey, U.S.A.

(*Received* 1 *August* 1961)

SUMMARY

Extraction and fractionation of lipids from intact ellipsoidal and triangular forms of *Trigonopsis variabilis* showed both to be rich in lipid, the content in triangular forms being twice that in ellipsoidal forms. Lipid was chiefly present in bound form with which principally choline was associated in triangular cells, whilst in ellipsoidal cells ethanolamine and serine predominated. Very little unsaturated lipid was found in the triangular forms, whereas a threefold greater iodine value was noted with the ellipsoidal forms. Polysaccharides of both cellular forms were extracted and fractionated. The triangular forms were seemingly devoid of glucan or chitin but contained a reducing polysaccharide that formed an unusual copper complex and appeared to be a polymer of glucose and mannose. The ellipsoidal forms contained a small amount of glucan and considerable amounts of glycogen. Cell walls were isolated from both triangular and ellipsoidal forms and found to consist principally of polysaccharide, nearly all of which was alkali-soluble. Walls of triangular forms contained somewhat more lipid than those of ellipsoidal forms, and phospholipid was twice that of ellipsoidal form walls.

INTRODUCTION

In a previous paper (SentheShanmuganathan & Nickerson, 1962*a*), the morphological development of *Trigonopsis variabilis* was shown to depend largely on the nutritional environment, especially as pertained to nutrition favouring elaboration of constituents of phospholipids. To obtain further evidence about the involvement of phospholipid synthesis in form development, lipids of whole organisms of both the triangular and ellipsoidal forms of *T. variabilis* have been examined. Striking differences were found between organisms of the two forms as to their contents of free and bound lipids, and the properties of their respective bound lipids. Cell walls were isolated from both cellular forms and the chemical composition of the isolated walls examined with a view to obtaining information permitting interpretation of cellular form in terms of its underlying molecular architecture.

* Present address: Medical Research Institute, Colombo 8, Ceylon.

METHODS

Microbiological methods. The organism used was *Trigonopsis variabilis* (Strain CBS 1040 obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, Holland). Growth medium and culture conditions used to obtain ellipsoidal or triangular cell forms were exactly as described in the preceding paper (Senthe-Shanmuganathan & Nickerson, 1962*a*). Organisms were harvested by centrifugation, washed twice with distilled water and taken for lipid analysis or for isolation of cell walls.

Isolation of cell walls. Freshly harvested, washed organisms were suspended in distilled water, and disrupted by agitation with glass beads in a Waring Blendor (cooled under flowing water) according to the technique of Lamanna & Mallette (1954). About 90 min. agitation was required to obtain complete breakage of organisms. At the end of this period, no whole organisms could be detected microscopically. Cell walls were separated from glass beads and particulate cellular debris by differential centrifugation and repeated washing alternatively with sucrose (8·5 %, w/v) or distilled water (see Falcone & Nickerson, 1956). To avoid contamination in later analyses, washing with phosphate buffer was omitted. After about thirty repetitions of washing and differential centrifugation, a fraction consisting exclusively of cell-wall material was obtained. This was suspended in water and lyophilized. White fluffy preparations were obtained by this procedure, and all the analytical results reported for cell walls are based on such material.

Lipid analysis of whole organisms and cell walls. The use of fresh cell material for lipid analysis is essential; most preliminary treatments cause more or less extensive hydrolytic and oxidative modifications of certain of the lipid fractions, particularly of the phospholipids (Peck, 1947). Therefore, freshly harvested organisms were used for the studies reported here. Unless otherwise mentioned, all extractions were carried out in an atmosphere of nitrogen obtained by bubbling oxygen-free nitrogen through solvents during all operations. Removal of solvents was effected by distillation under reduced pressure; the last traces of solvent were removed by flushing with N_2 gas. Diethyl ether, acetone, chloroform, and ethanol were used as lipid solvents, and were redistilled before use.

Free or readily extractable lipids of whole organisms or of cell walls were extracted by suspending organisms or walls in a mixture of ethanol + ether (1 + 1, v/v) and allowing to stand at room temperature under an atmosphere of N_2 for 5 days with occasional shaking. Solvent was separated by filtration and the process repeated twice. The extracts were combined, solvent removed, and residues exhaustively extracted with ether for 24 hr. Ethereal solutions were dried over anhydrous Na_2SO_4, and concentrated to small bulk under reduced pressure. A measured sample of concentrate was transferred to a weighed centrifuge tube, and the last traces of ether removed by flowing N_2. The residue was treated with acetone, and allowed to stand at 2° for 24 hr., whereupon phospholipids precipitated. The insoluble material was packed by centrifugation at 2° for 15 min at 19,600 *g*. The supernatant fluid was poured off, and adhering acetone drained off as thoroughly as possible. The last traces of solvent were removed in flowing N_2 and the residual material, taken as phospholipid, was weighed.

The residue obtained after the removal of free lipids with ethanol + ether, was

subsequently extracted twice with chloroform for a period of 24 hr. The chloroform extracts were combined to give a second fraction of readily extractable lipids.

Chemically bound and occluded lipids were extracted by subjecting residual material, after washing with aqueous ethanol (25 %, v/v), to a mild digestion with ethanol + ether (1 + 1, v/v) containing HCl (1 %, w/v) at 50° for 5 hr. (Crowder *et al.* 1936; Anderson *et al.* 1937). Cell residues were filtered off and extracted with ether and then with chloroform. All three extracts were combined, concentrated to an aqueous suspension, and extracted with ether for 24 hr. in a liquid–liquid extractor. The ethereal extract was dried over anhydrous Na_2SO_4, concentrated to small bulk, and made up to a known volume with ether. This fraction is referred to as total bound lipid. Chloroform- and acetone-insoluble fractions were determined on measured samples of total bound lipid, as described above for phospholipids.

Hydrolysis of lipids. To determine nitrogenous constituents of the bound lipids of whole organisms, the material was hydrolysed by refluxing for 3 hr. with 5 ml. 6N-HCl in methanol (Thannhauser, Benotti & Reinstein, 1939). Solvent and HCl were removed at 60° under reduced pressure to obtain a dry residue which was treated with water and again concentrated; this process was repeated twice. Finally, the dry mass was dissolved in a known volume of water and filtered in the cold to give a clear solution.

Selective hydrolysis of bound lipids was performed to distinguish the choline content of lecithin from that of sphingomyelin. Complete hydrolysis liberates choline from both lecithin and sphingomyelin, whilst the selective hydrolysis method of Schmidt, Benotti, Hershman & Thannhauser (1946) liberates choline from lecithin but not from sphingomyelin (Hack, 1947). For details of the method see Schmidt (1957).

Analytical methods. Choline was estimated by converting it to the insoluble enneaiodide with KI_3, dissolving the precipitate in ethylene dichloride, and determining optical density at 365 mμ (Appleton *et al.* 1953). Whenever an estimation was carried out, two standards were run side by side under the same conditions. Ethanolamine and serine were determined, by the colorimetric method of Axelrod, Reichenthal & Brodie (1953), after conversion into their coloured 2:4-dinitrofluorobenzene derivatives and separation on the basis of their differential solubilities in organic solvents. A mixture containing equal amounts of ethanolamine and serine was used side by side.

Total nitrogen content of bound lipids was determined by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943); NH_3 was distilled in the apparatus of Markham (1942) and trapped in the boric acid reagent of Conway & O'Malley (1942). Total phosphorus was determined by the colorimetric method of Fiske & SubbaRow (1925), described by Umbreit, Burris & Stauffer (1957), after digestion with concentrated H_2SO_4. Determinations of total reducing sugars of cell walls were carried out on acid hydrolysates by the anthrone method of Chung & Nickerson (1954); hexosamine was estimated by the Blix (1948) modification of the Elson & Morgan method (1933).

Paper chromatography. Amino acids in cell walls were determined by two-dimensional paper chromatography after cell walls had been hydrolysed with 6N-HCl for 24 hr. in a sealed tube at 100° under N_2. Whatman No. 1 paper was used for chromatograms, which were irrigated for 24 hr. with phenol (containing

8-hydroxyquinoline, 1 mg./2 ml.) + ammonia in one direction. Papers were then dried at 85° for 15 min. to remove phenol and were run in the other direction for 18 hr. with butanol + acetic acid + water (2 + 1 + 2, vol.). Amino acids were detected by spraying with ninhydrin (0·25 %, w/v) in water-saturated butanol and drying papers at 85° for 10 min.

Definition of terms used. Saponification number (SV) is the number of mg. of KOH neutralized by fatty acids, free or combined, in 1 g. lipid when the latter is completely saponified with ethanolic KOH. Iodine number (IV) is the number of g. iodine taken up by 100 g. lipid. Acid number (AV) is the number of mg. of KOH required to neutralize free fatty acids in 1 g. lipid. Ester number (EV) is obtained by difference on subtracting the acid value from the saponification value.

Determination of the above values. The methods described by Baldwin & Bell (1955) were employed. The iodine number of a lipid was determined as follows. To lipid dissolved in chloroform was added 1·0 ml. of pyridine sulphate dibromide reagent; the mixture was shaken thoroughly and allowed to stand at room temperature for 5 min. Residual bromine was determined by titration of iodine liberated from KI with a standard solution of sodium thiosulphate, with starch as indicator. Unsaponifiable material in lipids was estimated as follows. After saponifying a lipid with ethanolic KOH, it was extracted with ether for 5 hr. in a liquid–liquid extractor. The ethereal extracts were dried over anhydrous sodium sulphate, the ether removed, and the residue weighed.

Tests for steroids. Unsaponifiable material was dissolved in chloroform and the following tests performed. Salkowski reaction with conc. H_2SO_4 and Liebermann-Burchard reaction with acetic anhydride and conc. H_2SO_4 (Baldwin & Bell, 1955).

RESULTS

Electron photomicrographs of cell walls of the triangular form of *Trigonopsis variabilis*, prepared as described above, are shown in Pl. 1. The walls appear to be homogeneous (not fibrillar or granular), and essentially free of adhering intracellular material.

Carbohydrate composition

Acid hydrolysis of cell walls resulted in rapid dissolution of the suspension, accompanied by liberation of reducing substances. As hydrolysis proceeded, samples were withdrawn every 30 min. and centrifuged; hexosamine and reducing sugars were estimated in the clear supernatant fluid. It is seen from Fig. 1 that hydrolysis appeared to be complete within 60 min. for both triangular and ellipsoidal wall preparations. When refluxing was continued beyond 90 min., browning of hydrolysates occurred, with a loss of 15 % (w/v) of the estimated reducing carbohydrate. Although the hexosamine content of cell walls of both forms were the same (1·9 %, w/v), total carbohydrate values, as glucose, differed appreciably. With the ellipsoidal form, carbohydrate was 91 % whereas in the triangular walls, it was 81 % (Table 1).

Amino acid composition

Amino acid content of the triangular form walls was almost twice that of the ellipsoidal form walls, as judged by the intensity of ninhydrin spots on paper chromatograms. This agrees with total nitrogen content of the cell walls. As shown in

Table 2, glycine, threonine and alanine were present in major amounts in both forms. Next in order of magnitude were valine, leucine or isoleucine, glutamic and aspartic acids. Lysine was not detected in the hydrolysate of ellipsoidal walls while triangular walls gave only a faint spot. Falcone & Nickerson (1956) obtained evidence for the presence of fifteen amino acids in the cell walls of baker's yeast; similar findings were reported by Eddy (1958); and quantitative analyses of the amino acid composition of proteins in the walls of *Candida albicans* and of baker's yeast were presented by Kessler & Nickerson (1959).

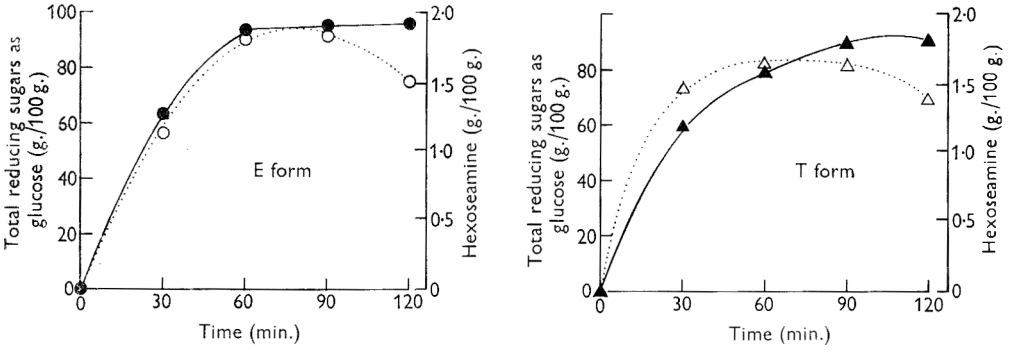


Fig. 1. Release of reducing sugars and hexosamine during acid hydrolysis of cell walls of ellipsoidal and triangular forms of *Trigonopsis variabilis*. 30 mg. cell-wall material hydrolysed with 15·0 ml. 2N-HCl at 100°; samples withdrawn at intervals and centrifuged; total reducing sugar (as glucose) and hexosamine were estimated in the supernatant by methods described in the text. O·····O = Total carbohydrate (as glucose) in cell walls of ellipsoidal form. ●——● = Hexosamine of ellipsoidal walls. △·····△ = Total carbohydrate (as glucose) in cell walls of triangular forms. ▲——▲ = Hexosamine of triangular walls.

Table 1. *Composition of cell walls of the ellipsoidal and triangular forms of* Trigonopsis variabilis

	Cell form	
	Ellipsoidal	Triangular
	mg./100 mg. dry wt. organism	
Moisture	3·0	3·0
Total carbohydrate (as glucose)	91·0	80·6
Hexosamine	1·91	1·87
Nitrogen	1·14	2·34
Phosphorus	0·086	0·153
N:P ratio	13·2	15·3
Free lipids:		
(*a*) Ether soluble	0·60	1·9
(*b*) $CHCl_3$ soluble	0·12	0·08
Bound lipids:		
(*a*) Total	4·7	6·7
(*b*) Acetone insoluble (phospholipids)	0·63	1·4

See text for the methods of isolation of lipids.

Nitrogen and phosphorus

Total nitrogen and phosphorus contents of cell walls of both forms of *Trigonopsis variabilis* are given in Table 1. Not only the nitrogen content, but also the phosphorus of the triangular walls was almost twice that of the ellipsoidal walls. Thus, the N:P ratios of ellipsoidal and triangular walls were 13:2 and 15:3, respectively. In cell walls of baker's yeast, Falcone & Nickerson (1956) reported a phosphorus value of 0·34 %. This higher value may reflect contaminating traces derived from phosphate buffers used in washing of wall preparations. In the present work, this possibility can be excluded since washing with phosphate was avoided. Values of 0·086 % phosphorus (with ellipsoidal walls) and 0·156 % (with triangular walls) were obtained.

Table 2. *Amino acid content of cell walls of ellipsoidal and triangular forms of* Trigonopsis variabilis *by chromatography*

The chromatogram papers were irrigated for 18 hr. with phenol + water + ammonia soln. (sp.gr. 0·880) (80 + 20 + 1 vol.) in one direction. The papers were dried, solvent removed, and irrigated in the other direction with butanol + acetic acid + water (2 + 1 + 2 vol.). The spots were located by spraying with 0·25% (w/v) ninhydrin in water-saturated butanol. Numbers give relative amounts of each amino acid.

Amino acids	Cell form: Ellipsoidal (Relative amount)	Cell form: Triangular (Relative amount)
Aspartic acid	1	2
Glutamic acid	2	4
Glycine	4	8
Threonine	4	8
Alanine	4	8
Lysine	—	1
Arginine	1	2
Tyrosine	1	2
Valine	2	4
Leucine or isoleucine	2	4

Distribution of phosphorus in cell-wall fractions

To ascertain the distribution of phosphorus in various fractions, cell walls of *Trigonopsis variabilis* were first freed from lipids (free and bound) and then fractionated. To obtain comparable data, the phosphorus content of lipid-free whole organisms was also estimated (Table 3). With lipid-free triangular walls, phosphorus was evenly distributed among the cold perchloric acid (PCA), hot PCA, hot NaOH, and residual fractions. On the other hand, the phosphorus content of ellipsoidal walls was highest in the NaOH-soluble fraction, whilst the other three fractions gave almost similar, lower, values. In fractionation of animal tissues (Schneider, 1945; Schmidt & Thannhauser, 1945), residual material soluble in hot NaOH (2 %, w/v) is usually regarded as phosphoprotein. More recently, Ågren (1958) showed that the 'phosphoprotein fraction' derived from baker's yeast or from *Lactobacillus casei*, indeed, contained phosphorylserine. With lipid-free whole organisms of both triangular and ellipsoidal forms of *T. variabilis*, phosphorus was found principally in the cold PCA extract, and least in the residual fraction. Both forms of whole cells gave almost identical values.

Nucleic acids

Barkulis & Jones (1957) reported the presence of nucleic acid in streptococcal cell walls. These authors mentioned that such nucleic acids were only released on digestion of cell walls with acid, and they did not observe an absorption peak at 260 mμ with aqueous suspensions. On the other hand, Salton & Horne (1951) and Falcone & Nickerson (1956) did not detect nucleic acids in cell walls of *Streptococcus faecalis* and baker's yeast, respectively. Eddy (1958) reported a value of 0·3 % for the nucleic acid content of yeast cell wall but questioned this figure, since his cell-wall preparation was not sufficiently clean, and attributed the finding to the small percentage of unbroken or partially broken cells which remained in the preparation, not to a component of the wall as such. However, isolation of nucleotide components from hydrolysates of clean cell walls of *Mucor rouxii* has recently been reported by Bartnicki-Garcia & Nickerson (1961).

Aqueous suspensions of cell walls, as well as cold and hot perchloric acid extracts of untreated cell walls and of lipid-free cell walls of both forms of *Trigonopsis variabilis* did not show any absorption peak at 260 mμ. Phosphorus in PCA extracts of cell walls of both cell forms (Table 3), therefore, did not derive from nucleic acid.

Table 3. *Distribution of phosphorus in the various fractions of lipid-free whole organisms and cell walls of the two forms of* Trigonopsis variabilis

The materials were suspended in 5 % (w/v) perchloric acid (PCA), kept at 5° for 30 min. and centrifuged. The residue was treated with 5 % (w/v) PCA, boiled for 30 min. at 100° and centrifuged. The sediment was washed twice with water and centrifuged. The residu was finally boiled with 2 % (w/v) NaOH and centrifuged.

Material	Fractionation procedure	Cell form: Ellipsoidal Phosphorus content (μg./100 mg.)	Cell form: Triangular Phosphorus content (μg./100 mg.)
Lipid-free whole organisms	None	120·6	133·0
	Cold PCA extract	64·0	67·0
	Hot PCA extract	20·0	19·4
	Hot NaOH extract	25·0	29·0
	Residue	13·6	15·8
Lipid-free cell walls	None	80·5	136·8
	Cold PCA extract	18·1	32·3
	Hot PCA extract	13·3	30·5
	Hot NaOH extract	28·8	36·9
	Residue	15·6	30·0
Cell walls	None	86·5	152·9
	Cold PCA extract	24·6	38·6
	Hot PCA extract	17·6	40·8
	Hot NaOH extract	25·2	33·6
	Residue	14·5	28·0

Lipids

Total lipids in cell walls of ellipsoidal and triangular forms of *Trigonopsis variabilis* were 5·42 % (w/v) and 8·68 % (w/v), respectively; the major portion of which occurred in a bound or occluded form, which was released only on digestion with

acidic ethanol+ether. The values obtained (Table 1) are comparable to those reported by Kessler & Nickerson (1959) for cell walls of baker's yeast. Intact organisms of *T. variabilis* were found to be unusually rich in lipids, and the contents of ellipsoidal and triangular forms differed appreciably. With the ellipsoidal forms, about 20 % of the cellular dry wt. was comprised of lipid, of which nearly all was present in a bound form. With triangular forms, the values were even higher: lipid comprised 43·7 % of the dry wt. of which the bulk was chemically bound (Table 4).

Table 4. *Lipid content of whole organisms of ellipsoidal and triangular forms of* Trigonopsis variabilis

	Cell form	
	Ellipsoidal	Triangular
Fraction	Amount (mg./100 mg. dry wt.)	
Free lipids:		
(*a*) Ether soluble	1·05	3·0
(*b*) $CHCl_3$ soluble	0·20	0·30
(*c*) Acetone insoluble fraction of (*a*)	Nil	Trace
Bound lipids	18·7	40·4

See text for the method of extraction of the lipids.

Table 5. *Fractionation of bound lipids of whole organisms of both forms of* Trigonopsis variabilis

	Cell form	
Bound lipids (g./100 g.)	Ellipsoidal	Triangular
(*a*) Acetone soluble	96·8	97·1
(*b*) Acetone insoluble (phospholipids)	3·2	2·9
(*c*) $CHCl_3$ insoluble	1·56	4·74
Nitrogenous constituents (mg./100 g.)		
(*a*) Total nitrogen	780·0	560·0
(*b*) Ethanolamine	70·3	38·6
(*c*) Choline	37·0	72·0
(*d*) Serine	66·0	36·0
(*e*) Choline from lecithin	8·4	29·0
Other properties		
(*a*) Saponification number	530	315
(*b*) Acid number	152	86
(*c*) Ester number (by difference)	378	229
(*d*) Iodine value	14·0	4·4
(*e*) Unsaponifiable matter	25 g./100 g.	20·1 g./100 g.
Phosphorus	453 (mg./100 g.)	244 (mg./100 g.)
N:P ratio	1·72	2·29
Colour test for sterols in unsaponifiable material	+	+ + +

Properties of bound lipids of whole cells and cell walls

Phospholipids, or the acetone-insoluble fraction of the bound lipids, comprised only a very small fraction of the bound lipid of whole organisms of *Trigonopsis variabilis*; ellipsoidal and triangular forms contained 3·2 and 2·9 %, respectively,

whilst the values for ellipsoidal and triangular walls were 13 and 21 %, respectively (Tables 1, 5).

Nitrogen and phosphorus contents of bound lipids of ellipsoidal forms were higher than observed with triangular forms (Table 5), whilst N and P contents of ellipsoidal walls were less than in triangular walls (Table 6). The N:P ratios for bound lipids, not only of whole organisms but also of triangular walls were higher than those observed with ellipsoidal forms. Analysis of nitrogenous constituents of bound lipids extracted from whole organisms revealed that triangular forms contained more choline than did the ellipsoidal forms, whilst ellipsoidal forms possessed more ethanolamine and serine. The use of a selective hydrolysis procedure which, presumably, released choline only from lecithin and not from sphingomyelin, showed that about 40 % of the choline in bound lipid of triangular forms was associated with lecithin, whereas only about 23 % of the choline of ellipsoidal forms was released (Table 5).

Table 6. *Analysis of bound lipids obtained from cell walls of ellipsoidal and triangular forms of* Trigonopsis variabilis

	Cell form	
	Ellipsoidal	Triangular
	Amount (mg./100 g. bound lipid)	
Analysis		
Nitrogen	120	420
Phosphorus	100	200
N:P ratio	1·2	2·1
Iodine no.	0	0

See text for the analytical methods used.

Other properties of lipids of the two cell forms also differed appreciably (Table 5), the most striking difference being observed in iodine numbers. Most of the fatty acids in triangular form lipids were found to be saturated, whereas those of ellipsoidal forms were unsaturated. In the preceding paper (SentheShanmuganathan & Nickerson, 1962*a*), we reported that the addition of oleic acid or glyceromono-oleate inhibited formation of triangular forms. The results shown in Table 5 are in agreement with the previous observation.

Unsaponifiable material obtained from bound lipids of triangular forms was found to contain almost three times more sterol than found in lipids of ellipsoidal forms (as estimated by the Liebermann & Burchard reaction). Absorption spectra of unsaponifiable material obtained from bound lipids of both forms (Fig. 2) exhibited a marked peak at 242 mμ. Paper chromatography, with propylene glycol + toluene as solvent system, revealed one spot that moved with the solvent front. Ultraviolet (u.v.) absorption spectra of bound lipids are shown in Fig. 3. With both triangular and ellipsoidal forms, a marked peak at 232 mμ is evident, suggesting that a major component of the unsaponifiable fraction is a steroid.

Polysaccharide composition of ellipsoidal and triangular forms of Trigonopsis variabilis

The method of Chung & Nickerson (1954) was used for the fractionation of polysaccharides of ellipsoidal and triangular forms of *T. variabilis* (Table 7). The triangular forms dissolved completely in hot KOH (30 %, w/v), leaving no insoluble material; this finding indicates that these forms were completely devoid of glucan or chitin. With ellipsoidal forms, however, small, but appreciable, amounts of glucan (0·55 μg./mg. cell dry wt.) were found. Moreover, the precipitate obtained with Fehling's solution dissolved completely on the first washing with NaOH (0·1 N), suggesting either that the cells were devoid of mannan, or that the 'copper mannan' precipitate was quite different from that obtained with baker's yeast.

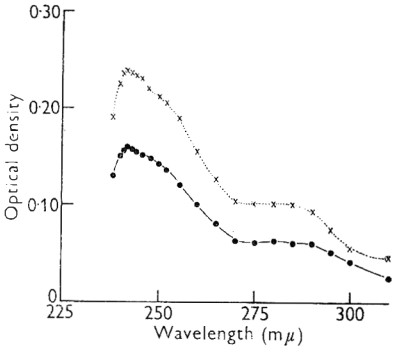


Fig. 2

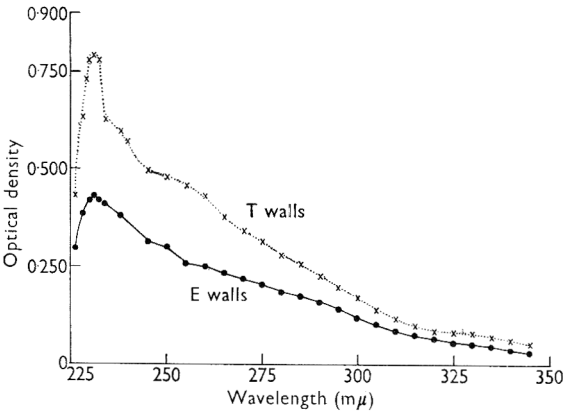


Fig. 3

Fig. 2. Absorption spectra of the unsaponifiable material obtained from bound lipids of whole cells of *Trigonopsis variabilis* (ellipsoidal and triangular forms). Equal amounts of material were dissolved in $CHCl_3$; absorption spectra measured against the solvent as reference. Note absorption maxima at 242 mμ. × · · · · · × = Unsaponifiable material from bound lipids of triangular forms. ●——● = Unsaponifiable material from ellipsoidal form lipids.

Fig. 3. Absorption spectra of bound lipids of cell walls of ellipsoidal and triangular forms of *Trigonopsis variabilis*. Bound lipids were dissolved in diethyl ether, and absorption spectra measured in cuvettes fitted with ground glass stoppers. Note absorption maxima at 232 mμ. × · · · · · × = Lipids of triangular walls. ●——● = Lipids of ellipsoidal walls.

Properties of polysaccharide soluble in KOH. A major portion of the total carbohydrate was found in the KOH fractions from both forms. Polysaccharide soluble in KOH was precipitated with 95 % (v/v) ethanol in water (after removal of lipid by extraction with ether), centrifuged and the supernatant fluid removed. The precipitate thus obtained was washed twice with ethanol, dissolved in water, and reprecipitated with ethanol. This procedure was repeated twice. A sample of the dry residue was dissolved in water and tested with Gram's iodine for glycogen. The polysaccharide obtained from the triangular forms reduced a large amount of iodine, and did not give the colour test for glycogen. On the contrary, polysaccharide from

the ellipsoidal forms did not exhibit reducing properties and gave a typical colour test for glycogen.

Both of these polysaccharides were soluble only with difficulty in hot water; insoluble residues remained even after boiling. Hydrolysis with N-H_2SO_4 did not give a clear solution; even after 16 hr. at 100° insoluble residues remained with the polysaccharides from both triangular and ellipsoidal forms. These hydrolysates were neutralized with $Ba(OH)_2$, and the precipitated $BaSO_4$ removed by centrifugation. The supernatant solutions were concentrated under vacuum and spotted on paper (Partridge, 1948). With ellipsoidal forms, glucose was identified, whilst with triangular forms mannose and glucose were found. No quantitative measurements were carried out. The polysaccharides from isolated cell walls of both forms behaved similarly.

Table 7. *Quantitative fractionation and analysis of polysaccharides of cell walls and whole organisms of the two forms of* Trigonopsis variabilis

The fractionations of the organisms were carried out as described by Chung & Nickerson (1954) and the carbohydrate contents estimated by the anthrone method of the same authors.

	Whole organism		Walls	
	Ellipsoidal	Triangular	Ellipsoidal	Triangular
Fraction	Amount (μg. glucose/mg. dry wt. organism or walls)			
Whole organisms or cell walls	440	405	920	820
Aqueous extract (25° for 30 min.)	0·77	5·4	66	17
Cold TCA* extract	19·30	6·2	23	7
Hot 30 % KOH material	321·0	358·0	809	780
Acetic acid extract	0·34	0	—	—
H_2SO_4 digest	0·55	0	—	—
Residue after KOH treatment	—	—	12	10
Alcohol precipitation of KOH soluble material				
Supernatant obtained after precipitation with 95 % EtOH	14·0	8·1	—	—
Acid hydrolysate of the ethanol precipitate	274·0	250·0	—	—
Insoluble residue obtained after hydrolysing the alcohol precipitate	60·0	46·2	—	—

* TCA = trichloroacetic acid.

DISCUSSION

Analyses of cellular components of ellipsoidal and triangular cell forms of *Trigonopsis variabilis* emphasize the enrichment of lipids in triangular cells, and the absence of structural constraints imposed by fibrillar wall components of polysaccharide nature. From studies in many laboratories the importance of the cell wall as determinant of cellular form has emerged. This concept is reinforced by the electron-micrograph shown herein (Pl. 1). The triangular or ellipsoidal cellular form results from the mould in which the wall is formed and not, primarily, from the intracellular environment. It must be admitted and emphasized at the outset, that we have been unable to obtain any information that would permit us to hold an opinion about the structural requirements for the triangular form. In the case of the

ellipsoidal form, we have advanced the opinion that a maximally disordered fibrillar structure (conferring a maximal modulus of elasticity) is requisite (Nickerson & Falcone, 1959; Falcone & Nickerson, 1959). Therefore, it is of importance to note that some glucan (fibrillar) component is found in ellipsoidal walls whereas such is not present in triangular walls.

As shown in the preceding paper (SentheShanmuganathan & Nickerson, 1962*a*), form development in *Trigonopsis variabilis* is subject to nutritional control. Substances such as methionine, that are methyl group donors, favour the development of populations of the triangular form. The role of methyl group donor substances in the biosynthesis of phospholipid moieties is well established, and mediation of the methionine effect on form development through promotion of phospholipid synthesis is strongly suggested by our analytical findings. Total lipid contents of both cellular forms of *T. variabilis* were high and in the triangular forms (43·7 %) was more than twice that in the ellipsoidal forms (20 %). In contrast, the total lipid contents of isolated cell walls (ellipsoidal = 5·42 %, triangular = 8·68 %) were in line with values available for baker's yeast. The proportion of phospholipid relative to total lipid in whole forms and in cell walls of *Trigonopsis* is most striking:

	Whole form	Cell wall
Ellipsoidal form	3·2	11·6
Triangular form	2·9	16·1

Not only are cell walls of the triangular form relatively richer in phospholipid, but the actual amount on a weight basis is twice that of the cell walls of the ellipsoidal form.

Physiological conditions which favour accumulation of lipid in most micro-organisms (e.g. continued incubation after the logarithmic phase of growth in a medium depleted in nitrogen-source, but containing an available carbon-source) are precisely those which lead to development of the triangular form of *Trigonopsis variabilis*. As emphasized in the preceding paper (SentheShanmuganathan & Nickerson, 1962*a*) triangular populations predominate only after incubation for more than 48 hr. in a medium which permits their development.

Thus, several lines of evidence favour the view that synthesis especially of phospholipids serves as a form-determinant in this yeast. Although such evidence as has been adduced points to the importance of the phospholipid of the cell wall in this connexion, further work is necessary to establish a relationship between the molecular form of such material and cellular shape.

REFERENCES

AGREN, G. (1958). On the nature of phosphorus linkages and biological functions of intracellular phosphoproteins. *Uppsala Universitets Årsskrift*, Nr. 5, pp. 5–64.

ANDERSON, R. J., REEVES, R. E. & STODOLA, F. H. (1937). The chemistry of the lipids of tubercle bacilli. LI. Concerning the firmly bound lipids of the human tubercle bacillus. *J. biol. Chem.* **121**, 649.

APPLETON, H. D., LADU, B. N., LEVY, Jun., B. B., STEELE, J. M. & BRODIE, B. B. (1953). A chemical method for the determination of free choline in plasma. *J. biol. Chem.* **205**, 803.

AXELROD, J., REICHENTHAL, J. & BRODIE, B. B. (1953). The direct determination of phosphatidyl ethanolamine and phosphatidyl serine in plasma and red blood cells. *J. biol. Chem.* **204**, 903.

BALDWIN, E. & BELL, D. J. (1955). *Cole's Practical Physiological Chemistry*, 10th ed., p. 101. Cambridge: W. Heffer and Sons Ltd.

BARKULIS, S. S. & JONES, M. F. (1957). Studies of streptococcal cell walls. I. Isolation, chemical composition and preparation of M protein. *J. Bact.* **74**, 207.

BARTNICKI-GARCIA, S. & NICKERSON, W. J. (1961). Composition and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Bact. Proc.* p. 94.

BLIX, G. (1948). The determination of hexosamines according to Elson & Morgan. *Acta chem. scand.* **2**, 467.

CHIBNALL, A. C., REES, M. W. & WILLIAMS, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354.

CHUNG, C. W. & NICKERSON, W. J. (1954). Polysaccharide synthesis in growing yeasts. *J. biol. Chem.* **208**, 395.

CONWAY, E. J. & O'MALLEY, E. (1942). Micro-diffusion methods. Ammonia and urea using buffered absorbents (revised methods for ranges greater than 10 μg N). *Biochem. J.* **36**, 655.

CROWDER, J. A., STODOLA, F. H., PANGBORN, M. C. & ANDERSON, R. J. (1936). The chemistry of the lipids of tubercle bacilli xliv. Comparative study of the lipids of the human tubercle bacillus. *J. Amer. Chem. Soc.* **58**, 631.

EDDY, A. A. (1958). The structure of the yeast cell wall. II. Degradative studies with enzymes. *Proc. Roy. Soc.* B, **149**, 425.

ELSON, L. S. & MORGAN, W. T. J. (1933). A colorimetric method for the determination of glucosamine and chondrosamine. *Biochem. J.* **27**, 1824.

FALCONE, G. & NICKERSON, W. J. (1956). Cell-wall mannan-protein of baker's yeast. *Science*, **124**, 272.

FALCONE, G. & NICKERSON, W. J. (1959). Enzymatic reactions involved in cellular division of micro-organisms. In *Biochemistry of Morphogenesis*, pp. 65–70. Ed. by W. J. Nickerson. London: Pergamon Press.

FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 735.

HACK, M. H. (1947). Estimation of the phospholipides in human blood. *J. biol. Chem.* **169**, 137.

KESSLER, G. & NICKERSON, W. J. (1959). Glucomannan-protein complexes from cell walls of yeasts. *J. biol. Chem.* **234**, 2281.

LAMANNA, C. & MALLETTE, M. F. (1954). Use of glass beads for the mechanical rupture of microorganisms in concentrated suspensions. *J. Bact.* **67**, 503.

MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.

NICKERSON, W. J. & FALCONE, G. (1959). Function of protein disulfide reductase in cellular division of yeasts. In *Sulfur in Proteins*, p. 409. Ed. by R. Benesch. New York: Academic Press Inc.

PARTRIDGE, S. M. (1948). Filter paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238.

PECK, R. L. (1947). The lipids of fungi with special reference to pathogenic fungi. In *Biology of Pathogenic Fungi*, p. 167. Ed. by W. J. Nickerson. New York: Ronald Press Co.

SALTON, M. R. J. & HORNE, R. W. (1951). Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. *Biochim. Biophys. Acta*, **7**, 177.

SCHMIDT, G. (1957). Determination of nucleic acids by phosphorus analysis. In *Methods in Enzymology*, vol. 3, p. 671. Ed. by S. P. Colowick & N. O. Kaplan, New York: Academic Press Inc.

SCHMIDT, G. & THANNHAUSER, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoprotein in animal tissues. *J. biol. Chem.* **161**, 83.

SCHMIDT, G., BENOTTI, J., HERSHMAN, B. & THANNHAUSER, S. J. (1946). A micromethod for the quantitative partition of phospholipide mixtures into monoaminophosphatides and sphingomyelin. *J. biol. Chem.* **166**, 505.

SCHNEIDER, W. C. (1945). Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

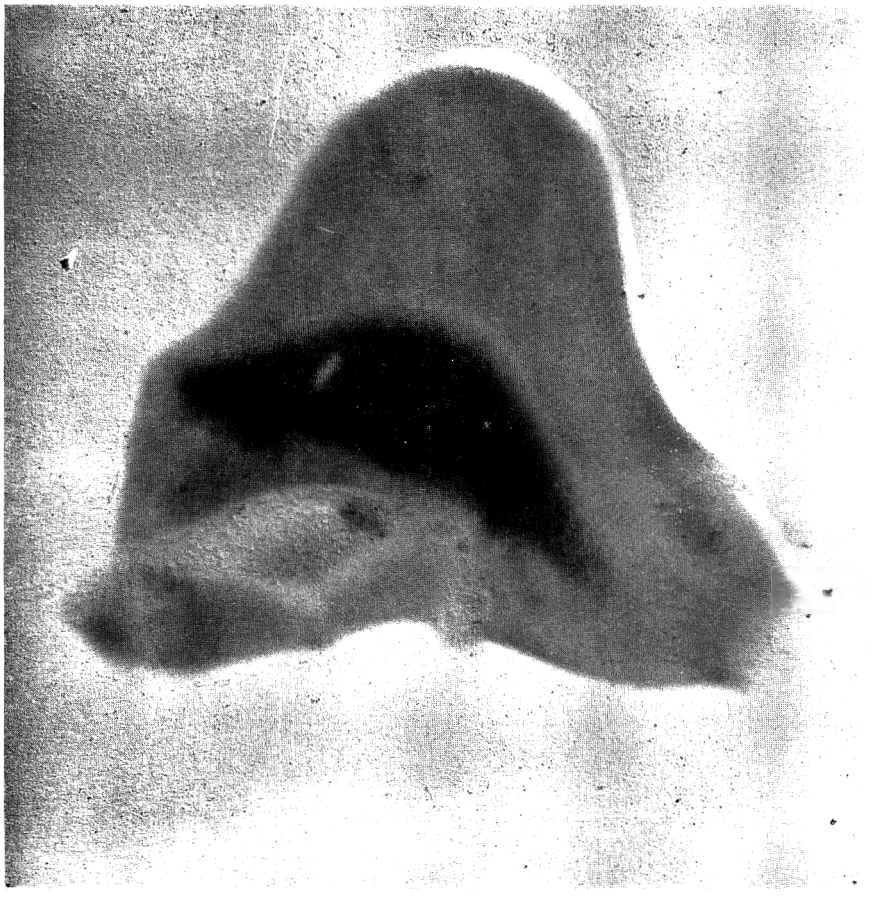
SENTHESHANMUGANATHAN, S. & NICKERSON, W. J. (1962*a*). Nutritional control of cellular form in *Trigonopsis variabilis*. *J. gen. Microbiol.* **27**, 437.

THANNHAUSER, S. J., BENOTTI, J. & REINSTEIN, H. (1939). Studies on animal lipids. XIV. The determination of lecithin, cephalin and sphingomyelin in body fluids and tissues with analyses of normal human sera. *J. biol. Chem.* **129**, 709.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*, 3rd ed., p. 273, Minneapolis: Burgess Publishing Co.

EXPLANATION OF PLATE

Electron micrograph of cell-wall preparation from triangular form of *Trigonopsis variabilis*. Magnification 21,750.



J. gen. Microbiol. (1962), **27**, 465-471
Printed in Great Britain

Transaminase and D-Amino Acid Oxidase of *Trigonopsis variabilis*

BY S. SENTHESHANMUGANATHAN* AND W. J. NICKERSON
Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey, U.S.A.

(*Received* 4 *May* 1961)

SUMMARY

The yeast *Trigonopsis variabilis* can utilize many single amino acids as sole source of nitrogen. Of the amino acids utilized, the D-isomers are oxidatively deaminated, whilst the L-isomers are transaminated with either α-ketoglutarate or pyruvate. The D-amino acid oxidase activity of this organism, when grown on DL-methionine as sole source of nitrogen, is the highest yet recorded, and may be recommended for the preparation of keto acids.

INTRODUCTION

It was shown in a preceding paper (SentheShanmuganathan & Nickerson, 1962*a*) that *Trigonopsis variabilis* can utilize a variety of single amino acids as sole source of nitrogen. The growth obtained with D- and L- methionine indicated that both the rate of growth and the total yield of organism with the D-isomer were almost twice that obtained with the L-amino acid. To elucidate the mechanism whereby amino acids are metabolized by this yeast, cell-free extracts were prepared from organisms grown with methionine or $(NH_4)_2SO_4$ as sole source of nitrogen. The data presented in this paper reveal the presence in this organism of extremely powerful D-amino acid oxidase and transaminase activities.

METHODS

Microbiological methods. Trigonopsis variabilis Schachner (CBS 1040) was used throughout this work. The culture medium and conditions of growth were as previously described (SentheShanmuganathan & Nickerson, 1962*a*).

Cell-free extracts. Cell-free preparations of the yeast were obtained from organisms grown in the medium containing salts, trace elements, glucose, biotin and thiamine. The nitrogen sources were DL-methionine or $(NH_4)_2SO_4$ at 25 mg. N/100 ml. The organisms were harvested by centrifugation, washed twice with distilled water, and suspended in 0·1M-phosphate buffer (pH 8·0) at a concentration equivalent to 1·5 g. dry wt. organism/20 ml. buffer. This suspension was subjected to sonic oscillation for 1 hr. in a Raytheon 50 W., 9 kc. magnetostriction oscillator at a plate voltage of 130 V. and an output voltage of 150 V. The suspension was then centrifuged for 15 min. at 19,600 *g* in the high speed angle-head of an International

* Present address: Medical Research Institute, Colombo 8, Ceylon.

refrigerated centrifuge. The clear supernatant fluid was dialysed against 0·01 M-KCl at 2° for 16–18 hr. with continuous stirring. The non-dialysable portion contained no debris and was used as the source of enzymes. The extracts from organisms grown with methionine or $(NH_4)_2SO_4$ as sole source of nitrogen will be referred to as extracts M and A, respectively. These were stored at −20° until required.

Reagents. The amino acids used were described in SentheShanmuganathan & Nickerson (1962*a*); 2-ketoglutarate and pyruvate were obtained from the California Corporation for Biochemical Research (Los Angeles 63, California, U.S.A.) and Schwarz BioResearch, Inc. (Mount Vernon, N.Y.), respectively. Solutions of the amino acids were made in 0·05 M-phosphate buffer (pH 8·0) whilst those of the keto acids were prepared in 1 % (w/v) Na_2CO_3 solution, adjusted to pH 8·0. Pyridoxal phosphate was purchased from the California Corporation for Biochemical Research.

Estimation of protein. The protein content of the preparations was determined by a turbidimetric method of SentheShanmuganathan (1960).

Assay of transaminase activity. The formation of glutamate or alanine from the amino acid and 2-ketoglutarate or pyruvate was taken as an index of transaminase activity. All incubations were made in duplicate in Thunberg tubes at 37°, gas phase N_2 (unless stated otherwise). Except where indicated, the complete system contained in a total volume of 3·0 ml.: pyridoxal phosphate (0·04 μmole), enzyme solution (0·1 ml.), and 2-keto acid (20 μmole) in 0·067 M-phosphate buffer (pH 8·0). The mixture was incubated for 10 min. before the addition of the amino acid (10 μmole L- or 20 μmole DL-amino acid). Two control systems were used, one without the amino acid and the other without the keto acid. At the end of the incubation period, 6·0 ml. ethanol were added and, after heating in a boiling water bath for 15 min., the denatured protein was removed by centrifugation. Equal volumes of the supernatant fluid were placed on paper chromatograms. The procedure of Woiwod (1949) was adopted for the separation of amino acids. After drying the papers, the positions of the amino acids were located by spraying with 0·1 % (w/v) ninhydrin in acetone (Dent, 1947).

Quantitative estimation of transaminase activity. The transamination between aspartic acid and 2-ketoglutarate was studied by measuring the change in absorption at 280 mμ in the Beckmann spectrophotometer (Cohen, 1955).

Assay of D-amino acid oxidase activity. The extent of amino acid oxidase activity was estimated by measurement of the amount of O_2 absorbed by the substrate (Krebs, 1951). The uptake of O_2 was followed manometrically in Warburg manometers by conventional techniques. Unless stated otherwise, all manometric experiments were carried out at 30°, in 0·05 M-phosphate buffer (pH 8·0) with air as gas phase. The centre well contained 0·2 ml. of 10 % (w/v) NaOH to trap any CO_2 produced. Reactions were initiated by the addition of the substrate from the side arm.

Growth measurements. Growth was measured in terms of dry weight, as described in SentheShanmuganathan & Nickerson (1962*a*).

RESULTS

The presence of transaminase activity in extracts prepared from organisms grown in methionine or in $(NH_4)_2SO_4$ as sole source of nitrogen, was tested with a variety of single amino acids as amino-group donors, and 2-ketoglutarate or pyruvate as amino-group acceptors. These estimations were carried out qualitatively by paper chromatography. Except with aspartic acid, no quantitative estimations were conducted. The results (Table 1) indicate that of the fourteen amino acids tested all, with the exception of proline, transaminated with 2-ketoglutarate in the presence of extracts A and M. In nine cases transaminase activity (glutamate formation) was observed with the DL form of the amino acid. In the other five cases the L form was active and it seemed likely that the L form of the DL mixtures might be the active component. This was tested and shown to be true with methionine since L-methionine but not D-methionine gave glutamate. When 2-ketoglutarate was replaced by pyruvate, only six of the fourteen acids (namely, α-aminobutyric acid, serine, threonine, tryptophan, tyrosine, valine) transaminated in the presence of either extract.

Table 1. *Transaminase activity in cell-free extracts of* Trigonopsis variabilis *prepared from organisms grown in methionine* (M) *or* $(NH_4)_2SO_4$ (A) *as sole source of nitrogen*

The complete system contained in a total volume of 3·0 ml.: pyridoxal phosphate (0·04 μmole), enzyme solution (0·1 ml.), and oxo acid (20 μmole) in 0·067 M-phosphate buffer (pH 8·0). The mixture was pre-incubated for 10 min. before the amino acid was added (10 μmole L or 20 μmole DL). Gas phase N_2; temp. 37°; incubation period, 60 min. Extract M from organism grown with methionine, extract A from organism grown with $(NH_4)_2SO_4$.

	Glutamate formation		Alanine formation	
Amino acid	Ext. M	Ext. A	Ext. M	Ext. A
None	−	−	−	−
DL-α-Alanine	+	+	−	−
DL-α-Aminobutyric acid	+	+	+	+
DL-Aspartic acid	+	+	−	−
L-Cysteine	+	+	−	−
L-Histidine	+	+	−	−
DL-Isoleucine	+	+	−	−
L-Leucine	+	+	−	−
DL-Methionine	+	+	−	−
L-Proline	−	−	−	−
DL-Serine	+	+	+	+
DL-Threonine	+	+	+	+
L-Tryptophan	+	+	+	+
DL-Tyrosine	+	+	+	+
DL-Valine	+	+	+	+

Transamination between aspartic acid and 2-ketoglutarate

To a quartz optical cell of the Beckmann spectrophotometer were added 0·1 ml. cell-free extract, 1·0 ml. 0·05 M-phosphate buffer (pH 8·0), DL-aspartic acid (20 μmole) and pyridoxal phosphate (0·04 μmole). After mixing, 2-ketoglutarate (20 μmole) was added and the change in absorption at 280 mμ was measured. Two controls were run simultaneously, one without the keto and the other without the amino acid. The results of such an experiment are shown in Fig. 1, where it is seen that both extracts A and M catalyse the transfer of amino group from aspartic acid

to 2-ketoglutarate. The rate of transamination with extract M was higher than that observed with extract A (0·060 μmole/min. as compared to 0·045 μmole/min.). When extract M was used, one of the control optical cells (which contained only aspartic acid) produced 0·01 μmole oxaloacetic acid/min. Such behaviour was not observed with extract A, implying that the extract M possessed a very slight amino acid oxidase activity.

Amino acid oxidase activity

The amino acid oxidase activity in extracts A and M was studied by measuring the O_2 uptake by the substrate amino acid. All experiments were carried out with the DL-isomers. Of the nineteen amino acids tested (Table 2), O_2 uptake was observed with fifteen of them, but the rates of O_2 absorption differed appreciably. With extract M, homocysteine was found to be the best substrate (415 μl. O_2/hr./mg. protein). When extract A was used, homocysteine was again found to be the best (136 μl. O_2/hr./mg. protein). With most of the amino acids studied, the rate of O_2 uptake in the presence of extract M exceeded (by more than three times) the rate observed with extract A.

Table 2. D-*Amino acid oxidase activity in cell-free extracts of* Trigonopsis variabilis *prepared from organisms grown in methionine* (M) *or* $(NH_4)_2SO_4$ (A) *as sole source of nitrogen*

The complete system contained in a total volume of 3·0 ml.: cell-free extract (0·3 ml.); amino acid (80 μmole); and 0·05 M-phosphate buffer (pH 8·0; 1·5 ml.); 10 % (w/v) NaOH (0·2 ml.) in the centre well. The enzyme preparation was added from the side arm when equilibrium was attained. Temp. 30°, incubation period, 60 min.

	μl. O_2 uptake/hr./mg. protein		
Amino acid	Extract M	Extract A	*Penicillium chrysogenum* (Krebs, 1951)
None	0	0	—
DL-Homocysteine	415	136	—
DL-Methionine	300	107	188
DL-nor-Valine	205	38	—
DL-α-Aminobutyric acid	201	55	134
DL-Valine	200	37	112
DL-Phenylalanine	172	19	126
DL-α-Alanine	153	31	146
DL-Leucine	151	30	208
DL-Tryptophan	122	6	38
DL-nor-Leucine	120	30	128
DL-Isoleucine	105	22	64
DL-Homocystine	104	28	—
DL-Tyrosine	86	19	—
DL-Serine	48	6	0
DL-Lysine	35	3	26
DL-Threonine	14	4	14
DL-Aspartic acid	8	1	0
DL-Ornithine	7	0	—
DL-α-Amino isobutyric acid	5	0	—

To learn whether the D, L, or both isomers were deaminated, four amino acids were tested in their L form (leucine, histidine, valine, phenylalanine). There was no uptake of O_2 with any of these amino acids in the presence of extracts A or M, indi-

cating that the amino acid oxidase was specific for the D-isomer only. For comparison, the D-amino acid oxidase activity obtained with *Penicillium chrysogenum* Q 176 (Krebs, 1951, based on data of Emerson, Puziss & Knight, 1950) is also given in Table 2. The results obtained with *Trigonopsis variabilis* run almost parallel to those obtained with *Penicillium chrysogenum*. This is the first time the presence of D-amino acid oxidase activity has been demonstrated in a yeast. The organisms grown with methionine as sole source of nitrogen are the richest source of amino acid oxidase found to date. *T. variabilis* may therefore be recommended as a potential source of D-amino acid oxidase for the preparation of keto acids.

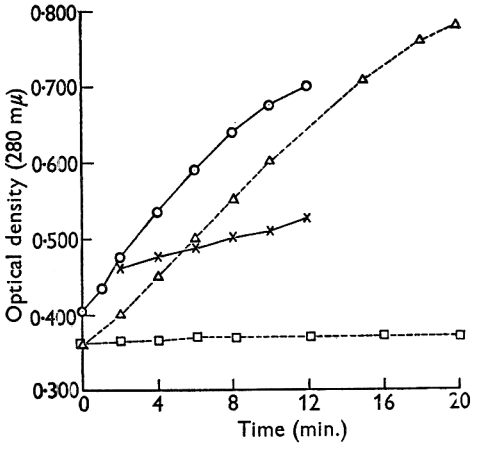


Fig. 1

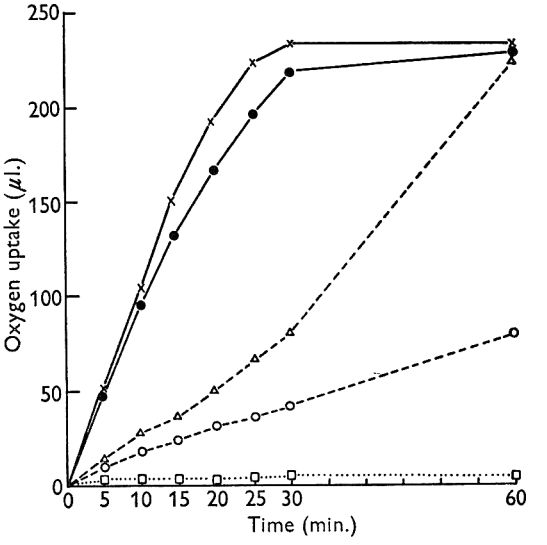


Fig. 2

Fig. 1. Transamination between 2-ketoglutarate and aspartic acid by cell-free extracts of *Trigonopsis variabilis* grown with DL-methionine or $(NH_4)_2SO_4$ as sole sources of nitrogen. The quartz cell of the Beckman spectrophotometer contained in a total volume of 3·0 ml.: extract (0·1 ml.), phosphate buffer (0·05 M; pH 8·0; 1·0 ml.), DL-aspartic acid (20 μmole); pyridoxal phosphate (0·04 μmole); 2-ketoglutarate (20 μmole). O——O Complete system containing extracts of methionine grown cells (M); ×——× complete system (M), less 2-ketoglutarate; △---△ complete system containing extracts of $(NH_4)_2SO_4$ grown cells (A); □---□ complete system (A), less 2-ketoglutarate.

Fig. 2. D-Amino acid oxidase activity of cell-free extracts of cells of *Trigonopsis variabilis* grown with DL-methionine or $(NH_4)_2SO_4$ as sole source of nitrogen. O_2 uptake measured in Warburg manometers. The complete system contained, in a total volume of 3·0 ml.: cell-free extract, 0·3 ml. = 1·5 mg. protein; 0·05 M-phosphate buffer (pH 8·0; 1·5 ml.); 10% (w/v) NaOH (0·2 ml.) in the centre well. Reaction was initiated by the addition of the amino acid (10 μmole) from the side arm; temp. 30°. ×——× Extract M, DL-methionine; ●——● extract M, DL-alanine; △---△ extract A, DL-methionine; O---O extract A, DL-alanine; □·····□ extracts A or M.

To follow the rate of oxidation of the amino acids, O_2 consumption was estimated manometrically at 10-min. intervals. For both D- and L-amino acids the rate of O_2 uptake with extract M was more than three times that obtained in the presence of extract A (Fig. 2). In 30 min. the substrates were completely deaminated by extract M, but incubation for 60 min. with extract A was required to achieve complete deamination of methionine and about one-third of the alanine. From

these results, and those in Table 2, it may be concluded that incubation of *Trigonopsis variabilis* with an amino acid as nitrogen source, can result in stimulation of deaminase activity by more than threefold.

To establish that the end product of the reaction between an amino acid and oxygen in the presence of the extracts was the keto acid corresponding to the amino acid (reaction 1):

$$CH_3—S—CH_2—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}—COOH + \tfrac{1}{2}O_2 \rightarrow CH_3—S—CH_2—CH_2—CO—COOH + NH_3$$

the product was isolated as the 2:4-dinitrophenyl hydrazone.

As a typical example, reaction (1) was examined in the case of methionine. When O_2 uptake ceased in the reaction flask, protein was precipitated with trichloroacetic acid (10 %, w/v, final concentration) and removed by centrifugation. The supernatant fluid was then extracted with ether and the ether extract dried with anhydrous Na_2SO_4. On removal of the ether, a thick syrupy liquid remained. This was treated with 5 ml. N-HCl and shaken with 10 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in N-HCl. After standing at 2° for 12 hr., the precipitated mass was filtered, washed with N-HCl and distilled water until the washings were colourless. The crude yellow product was recrystallized three times from ethanol + water until a constant melting point was obtained. The recrystallized product melted at 150°. The keto acid, α-keto-γ-methiolbutyric acid, corresponding to methionine, was also prepared according to the method described by Meister (1952) by using acetone powders of hog kidney cortex as source of D-amino acid oxidase. The 2:4-dinitrophenylhydrazone was also prepared from this keto acid as described above. The melting point was found to be 150° (Meister, 1957, m.p. 150°). A mixed melting determination also gave a value of 150°.

Table 3. *Growth of* Trigonopsis variabilis *under anaerobic conditions with* $(NH_4)_2SO_4$ *or* D-, L-, *or* DL-*methionine as sole source of nitrogen*

After inoculation, the flasks were flushed with N_2, stoppered with rubber stoppers carrying Bunsen valves, and incubated at 28° with continuous agitation for 72 hr.; medium at pH 6·0.

Nitrogen source	Growth after 72 hr. of incubation (mg. dry wt./l.)
None	5
$(NH_4)_2SO_4$	372
DL-Methionine	145
L-Methionine	135
D-Methionine	13

From these findings it is apparent that *Trigonopsis variabilis* metabolizes amino acids by transamination or by deamination. The D-isomer is deaminated oxidatively (reaction 2), whilst the L-amino acid is transaminated with either 2-ketoglutarate or pyruvate (reaction 3):

(2) D-Methionine + $\frac{1}{2}O_2$ → α-oxo-γ-methiol butyric acid + NH_3

(3) L-Methionine – 2-ketoglutarate or pyruvate → 2-keto-γ-methiol butyric acid + glutamic acid or alanine.

If only reactions 2 and 3 are available to the organism, then this yeast should be able to utilize only the L-isomer, and not the D-amino acid, in growth under anaerobic conditions. To test this supposition, $(NH_4)_2SO_4$ and D-, L-, or DL-methionine was used as sole nitrogen source in the medium previously described. After inoculation, the flasks were flushed with N_2, and incubated at 28°. At the end of 72 hr. of incubation, growth was measured in terms of dry weight of organism. In Table 3 it may be seen that with L- or DL-methionine growth was very much greater than that obtained with the D-isomer.

DISCUSSION

D-Amino acid oxidase activity has been demonstrated in cell-free preparations obtained from a number of filamentous fungi (including *Neurospora crassa*, *N. sitophila*, *Aspergillus niger*, and several species of *Penicillium*; see Krebs, 1951) and from a few bacteria. The enzyme has been found in liver and kidney of all vertebrates in which it has been sought. In view of the very high activity of this enzyme in our preparations from *Trigonopsis variabilis*, it is somewhat remarkable that this is, apparently, the first report on the presence of D-amino acid oxidase in a yeast. Since the recognition of the existence of separate enzyme systems for the oxidation of L- and of D-amino acids (Krebs, 1935) it is also somewhat remarkable that 'no satisfactory answer can be given to the question of the physiological significance of D-amino acid oxidases' (Krebs, 1951). The statement, apparently, holds good today. However, as Krebs pointed out in his review, D-amino acid oxidase is a useful chemical tool for the determination of the presence of D-amino acids, and for the preparation of α-keto acids from several of the α-amino acids. In *T. variabilis* there is provided an organism, easily grown, that is a rich source of D-amino acid oxidase.

This work was supported in part by grants from the National Institutes of Health, U.S. Public Health Service. One of us (S.S.) was Pfizer Postdoctoral Fellow in Microbiology, on leave of absence from the Medical Research Institute, Colombo, Ceylon, when this work was done.

REFERENCES

COHEN, P. P. (1955). Estimation of animal transaminases. In *Methods in Enzymology*, vol. 2, p. 178. Ed. by S. P. Colowick & N. O. Kaplan. New York: Academic Press Inc.

DENT, C. E. (1947). The amino-aciduria in Fanconi Syndrome. A study making extensive use of techniques based on paper partition chromatography. *Biochem. J.* **41**, 240.

EMERSON, R. L., PUZISS, M. & KNIGHT, S. G. (1950). The D-amino acid oxidase of molds. *Arch. Biochem.* **25**, 299.

KREBS, H. A. (1935). Metabolism of amino-acids. III. Deamination of amino acids. *Biochem. J.* **29**, 1620.

KREBS, H. A. (1951). Oxidation of amino acids. In *The Enzymes*, vol. 2, part 1, p. 499. Ed. by J. S. Sumner & K. Myrbäck. New York: Academic Press Inc.

MEISTER, A. (1952). Enzymatic preparation of α-keto acids. *J. biol. Chem.* **197**, 309.

MEISTER, A. (1957). *Biochemistry of the Amino Acids*, p. 79. New York: Academic Press Inc.

SENTHESHANMUGANATHAN, S. (1960). The purification and properties of the tyrosine-2-oxo-glutarate transaminase of *Saccharomyces cerevisiae*. *Biochem. J.* **77**, 619.

SENTHESHANMUGANATHAN, S. & NICKERSON, W. J. (1962*a*). Nutritional control of cellular form in *Trigonopsis variabilis*. *J. gen. Microbiol.* **27**, 437.

WOIWOOD, A. J. (1949). A method for the estimation of micro amounts of amino nitrogen and its application to paper partition chromatography. *Biochem. J.* **45**, 412.

J. gen. Microbiol. (1962), **27**, 473–476
Printed in Great Britain

A Study of Some Mutations in a Strain of *Rhizobium trifolii*

By B. M. GUPTA* and J. KLECZKOWSKA
Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 12 *July* 1961)

SUMMARY

Mutants of a strain of *Rhizobium trifolii* were obtained by selective action of bacteriophage and streptomycin and by exposure to ultraviolet (u.v.) radiation. All mutants resistant to bacteriophage were streptomycin-susceptible and all resistant to streptomycin were bacteriophage-susceptible; no survivor after exposure to u.v. radiation resisted either the bacteriophage or streptomycin. Mutation to bacteriophage resistance was closely correlated with inability to fix nitrogen in symbiosis with red clover. Some streptomycin-resistant mutants used streptomycin as a nutrient supplement, enabling them to grow on a mineral medium without growth factors. All but two mutants remained serologically indistinguishable from the original strain.

INTRODUCTION

Previous work on mutation among nodule bacteria was mostly concerned with changes in the ability to form nodules on the leguminous host and in the effectiveness of nitrogen fixation in the nodules (briefly reviewed by Kleczkowska, 1950). Kleczkowski & Thornton (1944) found that an ineffective mutant of *Rhizobium trifolii* and its effective parent were indistinguishable serologically. Kleczkowska (1950) found that phage-resistant mutants also tended to differ from parental strain in nitrogen-fixing efficiency. It seemed, therefore, that mutation in susceptibility to a bacteriophage is correlated with the symbiotic effectiveness, although the degree of correlation varied from one bacterial strain to another. Some of the mutants also differed in colony morphology and pigmentation, and analysis showed that changes in colony morphology, pigmentation or effectiveness were independent. This work investigates the problem further by comparing mutants selected by bacteriophage or streptomycin treatment with those caused by u.v. irradiation. The nutrient requirements and serological specificity of all strains were also examined.

METHODS

The strain of *Rhizobium trifolii* used in this work, strain A, has been continuously used over many years and has always been effective in fixing nitrogen. It was periodically re-isolated from a single colony.

* Present address: Microbiology Department, Central Drug Research Institute, Lucknow, India.

Two different media were used: (1) Yeast water mineral salts mannitol agar (YWA); (2) a mineral salts medium with galactose (MSG). The first medium was the same as used by Kleczkowska (1950). The composition of the MSG medium is: 0·3 g. KH_2PO_4; 0·7 g. Na_2HPO_4; 0·3 g. $(NH_4)_2SO_4$; 0·1 g. $MgSO_4 \cdot 7H_2O$; 0·06 g. $CaCl_2$. The salts were dissolved in 800 ml. H_2O and the solution adjusted to pH 7·2. The solution was sterilized at 121° for 15 min. and mixed with 200 ml. of a separately sterilized 1%(w/v) solution of galactose. To both media 1·2%(w/v) agar was added and plates were prepared each containing 10 ml. medium. The MSG medium was used to study nutrient requirements of mutants. The original *Rhizobium trifolii* strain A grows well on the YWM medium but on the MSG medium only after adding biotin and thiamine.

The bacteriophage was a mutant of phage S_2P_{11} originally isolated from soil (Kleczkowska, 1945). Filtered lysed rhizobium cultures in liquid YWM medium were used for stock. They contained about 10^9 phage particles/ml. (by plaque count). To obtain phage-resistant and streptomycin-resistant mutants, plates containing phage or streptomycin were inoculated with water suspension of bacteria. Phage stock was added to the liquid media at 42° in the amount corresponding to 1·0 ml./plate just before the medium was poured into Petri dishes. In addition to this, phage stock was mixed in equal volumes with bacterial suspension before inoculating the plates. Streptomycin sulphate was added to the media at 0·5 mg./plate. The concentration of bacteria in the suspension, usually about 2×10^8 bacteria/ml., was determined by haemocytometer and by plating. To inoculate the plates, 0·1 ml. of the bacterial suspension was placed on the surface of agar medium and spread evenly with a platinum rod.

Bacterial colonies that developed on plates with bacteriophage or with streptomycin after several days incubation at 25°, were counted and isolated for further work.

Ultraviolet irradiation was done by placing 5 ml. of a thin water suspension of bacteria (about 10^5 bacteria/ml.) on a Petri dish of 9 cm. diameter and exposing the dish for 1 min. to a Hanovia low-pressure mercury lamp. The intensity of the radiation was about 1500 μW./cm.^{2}. The lamp was fitted with a filter to exclude radiation below 240 mμ, so that most of the radiation was of a wavelength of 254 mμ. The fluid was rocked during irradiation to equalize mutual shading. After irradiation a number of YWM agar plates were inoculated with 0·1 ml. of the irradiated suspension spread evenly with a platinum rod. The colonies that developed after incubation for a few days at 25° were counted and isolations made for further work. About 0·5% of the bacteria treated survived irradiation.

The effectiveness in nitrogen fixation in root nodules was tested on late-flowering Montgomeryshire red clover as previously described (Kleczkowska, 1950).

The nutrient requirements of bacteria were tested on MSG agar plates supplemented with test nutrients. One ml. of bacterial suspension (containing about 10^9 bacteria) was added to 10 ml. melted agar medium previously cooled to 42° and the mixture poured into a Petri dish. The plates were then divided into radial segments and a drop of solution of a supplementary nutrient was placed in the middle of each segment. The following solutions were tested: 0·01% (w/v) biotin; 0·01% (w/v) thiamine; 0·01% (w/v) yeast nucleic acid; a solution containing 0·1% (w/v) each of asparagine and glutamine. When mixtures were used each component was present at the above concentrations.

RESULTS

Table 1 shows that none of the strains obtained by the selective action of streptomycin resisted bacteriophage and none selected by bacteriophage resisted streptomycin. These two kinds of mutation are, therefore, independent. Mutation to inability to fix nitrogen was much more frequent among phage-selected than among streptomycin-selected mutants and the χ^2 test showed that the incidence of changes in effectiveness in the two kinds of mutant differs significantly, $P < 0{\cdot}001$.

No similar comparison can be made with survivors after u.v. irradiation, as it is not known what proportion of them were mutants. There were certainly 3 mutants out of the 23 isolates, 2 of which were ineffective in nitrogen fixation and the third altered in a nutrient requirement (see below). Any of the others may or may not have been mutants in some other respects that were not tested. None of the tested strains become resistant either to the bacteriophage or to streptomycin. Certainly u.v. irradiation did not prove to be an efficient method for obtaining mutants in the characters under test.

Table 1. *A comparison of some properties of the original bacterial strain A of* Rhizobium trifolii *with those of survivals after three different treatments*

			Streptomycin		Bacteriophage		Symbiotic effectiveness in nitrogen fixation	
			R	S	R	S	E	I
Pretreatment	Proportion of survivals	Total numbers of isolates tested	No. strains isolated from single colonies that fell into indicated categories					
No treatment (original strain A)	—	20	0	20	0	20	20	0
Streptomycin	2×10^{-8}	28	28	0	0	28	27	1
Bacteriophage	3×10^{-3}	20	0	20	14	6	6	14
U.v. irradiation	5×10^{-3}	23	0	23	0	23	21	2

R = resistant; S = susceptible; E = effective; I = ineffective.

Table 1 shows that 6 isolates after phage treatment proved on subcultivation to be susceptible to the phage, whereas 14 remained resistant. Similar results were obtained and discussed previously (Kleczkowska, 1950). Four ineffective mutants occurred among the 6 reverted susceptible strains, and 9 among the 14 which remained phage resistant. The ineffective mutants were about equally distributed between these two groups.

Neither the original bacterial strain A nor those isolated after these treatments grew on the MSG medium, and nor when this was supplemented with yeast nucleic acid, a mixture of amino acids, or with biotin. All strains grew on the YWM medium and in the MSG medium supplemented either with yeast extract or with biotin + thiamine. Only one mutant obtained after u.v. irradiation grew on the basal medium supplemented with thiamine only. Thus the search for mutants in nutrient requirements by means of the set of supplements used in the work proved unfruitful, but 10 mutants were obtained for which streptomycin appeared as a nutrient supplement in the presence of which they could grow on the MSG medium.

The mutants which grew on the basal medium + streptomycin were amongst those that were obtained by exposing the original strain to streptomycin. Streptomycin-resistant mutants were obtained both on the YWM medium and on the MSG medium and the proportion of survival was about the same on each medium. Ten of the streptomycin-resistant mutants shown in Table 1 were obtained on MSG medium and 18 on the YWM medium. The single ineffective mutant was isolated from YWM medium.

The mutants obtained on the MSG medium grew normally on this medium with streptomycin but not without. They retained this property after several subcultures on YWM medium without streptomycin. The mutants obtained on YWM medium with streptomycin grew normally only on this medium (with or without streptomycin). They did not grow on MSG medium without streptomycin; in its presence they formed incipient colonies.

All strains were tested with an antiserum prepared against the original strain A. All agglutinated except two obtained by phage treatment. One of these remained permanently phage-resistant and was also ineffective in nitrogen fixation, the other reverted to phage susceptibility and was effective.

DISCUSSION

The strain A of *Rhizobium trifolii* used in this work was effective in nitrogen fixation, required biotin and thiamine as nutrients and was susceptible to streptomycin and phage. The study of changes in its properties showed a high degree of correlation between mutation to phage resistance and to ineffectiveness in nitrogen fixation. (The phage-resistant mutants contained 71% ineffective strains.) Previous work on two ineffective and phage-susceptible strains showed no effective mutants among the phage-resistant strains of one strain. The other produced 7% of mutants which were both phage-resistant and effective; but the degree of correlation was much lower (Kleczkowska, 1950) than with strain A. The problem now is whether the apparent ease with which effective strain mutated to ineffectiveness is the general rule; obviously too few strains have yet been tested to allow any general conclusion.

We thank Dr P. S. Nutman for his interest in this work.

REFERENCES

KLECZKOWSKI, A. & THORNTON, H. G. (1944). A serological study of root nodule bacteria from pea and clover inoculation group. *J. Bact.* **48**, 661.

KLECZKOWSKA, J. (1945). The production of plaques by Rhizobium bacteriophage in poured plates and its value as counting method. *J. Bact.* **50**, 71.

KLECZKOWSKA, J. (1950). A study of phage resistant mutants of *Rhizobium trifolii*. *J. gen. Microbiol.* **4**, 298.

J. gen. Microbiol. (1962), **27**, 477–488
With 1 plate
Printed in Great Britain

Properties and Behaviour of a Virus Depending for its Multiplication on Another

By B. KASSANIS
Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 19 *July* 1961)

SUMMARY

The Rothamsted culture of tobacco necrosis virus contains two serologically unrelated viruses one of which, called the 'satellite virus' (SV), causes no lesions and multiplies detectably only when the other (TNV) is present. It decreases the size of necrotic local lesions formed by TNV. Inocula containing both viruses gave the same, fewer or more lesions than inocula containing only TNV ; the results depended on the ratio of the two viruses in the mixed inoculum, on the species and age of the test plants and, particularly, on the temperature at which the plants were kept after the inoculation. The concentration of TNV decreased when SV was also present; the extent of the decrease depended on the ratio of the two viruses in the inoculum and other factors. Inoculation with SV 5 days before inoculation with TNV still decreased lesion size and led to the multiplication of SV. Phenol-disrupted SV affected lesion size only when inoculation with TNV followed within a few hours, but when inoculation with TNV was delayed by up to 2 days, SV sometimes multiplied detectably. SV has the smallest particle reported for any plant virus; diameter *c.* 17 mμ and weight equivalent to a molecular weight of about $1{\cdot}9 \times 10^6$, of which 20 % is nucleic acid. It is exceptionally stable, retaining infectivity after 17 years at 3° and after heating for 10 min. at 90°. TNV was much more susceptible to inactivation by heat and by ultraviolet light than SV. The two differed greatly in electrophoretic mobility and were readily separated from mixed preparations by electrophoresis.

INTRODUCTION

The Rothamsted culture of tobacco necrosis virus was long known to contain spherical particles of two sizes (Bawden & Pirie, 1942, 1950) but the significance of this was not understood until they were found to be serologically unrelated viruses, the smaller of which multiplies detectably only in the presence of the larger. The simultaneous multiplication of the two produces smaller necrotic local lesions in French bean than when the large particles are multiplying alone; and the ratio of large to small lesions falls as the proportion of small particles in the inoculum increases; a great excess of small particles can decrease the number of lesions (Kassanis & Nixon, 1960, 1961). The experiments now to be described show other effects of interaction between the two viruses and how their interactions depend on the species of host plant and changes in the environment. Also, by using the

proportion of large to small lesions formed by a mixed inoculum as a method of assaying small particle infectivity some of the properties of the small virus particles have been determined. For need of a better name, the virus with small particles was called 'satellite virus' (SV) because it is always in association with and dependent on the large virus particles.

METHODS

The virus inocula were prepared as described by Kassanis & Nixon (1961). The satellite virus (SV) was obtained from the top zone after centrifugation in sucrose gradients of purified preparations of an isolate of the Rothamsted culture. The ratio of small to large particles varied in different preparations, depending on the season and the age of the tobacco plants, from 10:1 to 250:1. The total yield of virus was smaller in summer than winter. The particles of two sizes separated into zones best when the preparations contained no more than 2 mg./ml. of virus. Plants inoculated with purified preparations of SV produced no lesions and the virus did not multiply detectably in them. SV can be activated by two tobacco necrosis viruses, TNVa or TNVb (Kassanis & Nixon, 1961), but in the present work TNVb was mainly used. All virus dilutions were in water. Purification, infectivity tests and particle counts with the electron microscope were made as before (Kassanis & Nixon, 1961).

The proportion of SV surviving after various treatments was measured by comparing its effect on the lesion formed by TNVb with untreated SV of known concentration. The treated SV was inoculated at one dilution and the untreated at 4 dilutions, after mixing with constant amounts of TNVb (enough to give about 20–60 lesions/half bean leaf). The numbers of large lesions were plotted against the concentration of untreated SV, and the concentration of SV remaining active in the treated preparation was estimated by interpolation. This method assumes that loss of the ability of SV to affect the size of lesions produced by TNVb coincides with loss of infectivity; there is evidence to show that the assumption is correct.

An antiserum to SV was prepared by injecting a rabbit with SV purified by two successive centrifugations in sucrose gradients. A total of 0·7 mg. of SV was injected into a rabbit in three injections; the first was intravenous and a month later two intramuscular injections were given on successive days. The virus injected intramuscularly was emulsified in Bacto Adjuvant, Complete (Difco Laboratories). The rabbit was bled a month after the last injection and when titrated against SV at 20 mg./l. the serum precipitated when diluted 1/400. This antiserum did not precipitate TNVa or TNVb; nor did antisera to these viruses precipitate SV, thus confirming earlier evidence that the large and small virus particles are serologically unrelated (Kassanis & Nixon, 1961).

The concentration of purified virus preparations was estimated from measuring their optical density at 260 mμ, and the concentration of the virus in sap by determining the highest dilution at which a specific precipitate was produced with the homologous antiserum. The sap used in serological tests was frozen, thawed, left for a day at 20°, centrifuged and heated at 45° for 10 min. before again being centrifuged at 8000 *g*. When the increase of virus in bean leaves was investigated, the water lost from the necrotic leaves was estimated by comparing their water content with that of uninoculated leaves and the loss was compensated for by

adding an appropriate amount to the leaves when they were ground before extracting the sap. Most of the experiments were made between autumn and spring when the glasshouse temperature fluctuated between 16° and 20°. The temperature in summer fluctuated between 20 and 30°, which sometimes was too high for virus multiplication.

RESULTS

Interference during lesion formation

The necrotic local lesions formed by inocula containing only TNVa or TNVb were all large, but inocula that also contained SV gave some small lesions, the number of which depended on the amount of SV in the inoculum (Fig. 1). The total number of lesions (large and small) formed by mixed inocula (constant TNVa or TNVb and varying amounts of SV) was sometimes the same over a wide range of concentrations of SV (Table 3 in Kassanis & Nixon, 1961), sometimes more and sometimes fewer (Fig. 1), depending on the age and species of the inoculated plants and their growing conditions before and after inoculation. A decrease was usual only with high concentrations of SV and when all the lesions were small, but in

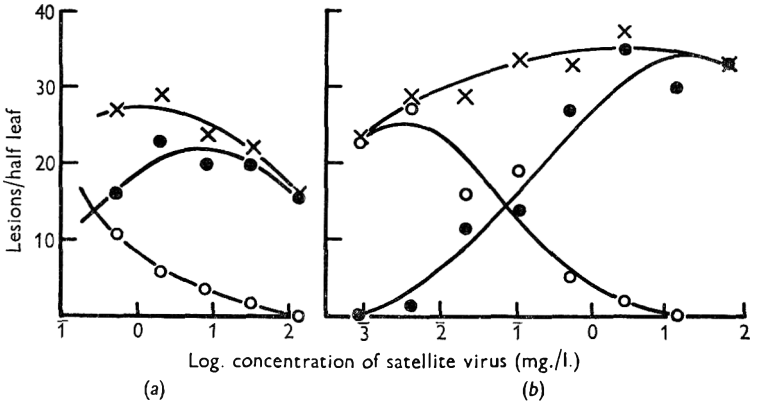


Fig. 1. Lesions/half leaf of French bean inoculated with mixtures of constant TNVb (0·3 mg./l.) and different amounts of SV. ×—×, total number of lesions; ○—○, large lesions; ●—●, small lesions. (*a*) and (*b*), experiments made at different times of the year.

summer it happened with lower concentrations of SV than in winter. Increases occurred mostly in winter and only when the ratios of the two viruses were such that not all lesions were small.

The number of lesions produced by a given inoculum of tobacco necrosis virus depends on the temperature at which the plants are kept (Kassanis, 1952). An experiment was therefore made to find whether the temperature also affected the extent to which TNVb and SV interacted. Bean plants were inoculated with constant amounts of TNVb mixed with varying amounts of SV and placed in glass chambers kept at 17°, 22° and 26°. The lesions appeared 1 day sooner at 26° than at the lower temperatures, but were fewer and all large. The highest concentration of SV decreased lesion numbers at all temperatures, and did so more at higher than at lower temperatures (Table 1). It is interesting that, with 1 mg./l. of SV, the total number of lesions decreased at 26°, but increased at 22° and even more at 17°.

These results strongly suggest that the interactions between TNVb and SV differ at different seasons mainly because of difference in temperature.

From these results it can be argued that, for a lesion to form, at least one of the susceptible sites (situated in one or more neighbouring cells) has to be occupied by TNVb. But when the concentration of SV is high or when the susceptible sites are few (by keeping the plants at a high temperature after inoculation or by other treatments), then the chances are that all the sites will be occupied by SV and the lesion will not be formed. The fact that with susceptible plants kept at relatively low temperatures considerably higher concentrations of SV were needed to decrease the total number of lesions suggests that each infectable centre may contain several susceptible sites.

Table 1. *Effect of temperature on number of large and small lesions formed by tobacco necrosis virus (TNVb) simultaneously inoculated with satellit evirus (SV) at different concentrations*

Concentration of SV* (mg./l.)	Small lesions			Large lesions			Total of small + large lesions		
	17°	22°	26°	17°	22°	26°	17°	22°	26°
	Number of lesions from 8 bean-half leaves								
25	227	93	0	0	0	4	227	93	4
5	915	614	0	94	4	22	1009	618	22
1	1097	522	0	113	33	36	1210	555	36
0·2	333	299	0	259	189	74	592	488	74
0·04	139	116	0	558	384	180	697	500	180
0·008	38	13	0	513	341	137	551	354	137

* Mixed with TNVb at 0·3 mg./l.

The interaction between TNVb and SV is also influenced by the age of the plants and the conditions in which they are kept before inoculation. When bean plants of two ages were inoculated with the same inocula (1·2 mg./l. of TNVb mixed with different amounts of SV), the large lesions expressed as a percentage of the small were 5, 18, 59 in young plants and 9, 38 and 138 in old plants, respectively, for 2·4, 0·6 and 0·15 mg./l. of SV, suggesting that, as plants age, their resistance to infection by SV increases more than to TNVb.

The susceptibility of the plants to virus infection can be increased by keeping them at 36° for a day or two before inoculation (Kassanis, 1952). To see whether their preinoculation treatment affects the interaction between the two viruses, bean plants were kept for one day at 36° or 18° and then inoculated on opposite half leaves with TNVb alone and TNVb mixed with SV (0·25 mg./l. for each virus). The plants pretreated at 36° produced about twice as many lesions as plants kept at 18° before inoculation. The presence of SV increased the total number of lesions on both lots of plants, by 42 % in plants pretreated at 36° but by only 17 % in plants kept at 18°, suggesting that the pretreatment increases susceptibility to infection by SV more than by TNVb. The interaction between SV and TNVb also depends on the species of plant. In *Nicotiana glutinosa* L. lesions look the same whether inoculated with TNVb alone or mixed with SV. When bean and *N. glutinosa* plants were inoculated on opposite half leaves with TNVb alone (0·6 and 6 mg./l., respectively, for the two species) or together with SV (10 mg./l.), the two inocula produced the

same number of lesions in beans, whereas in *N. glutinosa* the mixed inoculum produced on average only 14% of the number of lesions produced by TNVb alone. Hence concentrations of SV that do not affect the number of lesions produced by TNVb in beans have a large effect in *N. glutinosa*.

Table 2. *Numbers and ratios of large and small lesions when mixed inocula (TNVb and SV) are inoculated at different dilutions*

Inocula	Size and number of lesions on 6 half-leaves of French bean: Large	Small	Large/small
Purified preparations (TNVb 0·8 mg./l. and SV 0·6 mg./l.)			
Undiluted	107	50	2·1
Diluted 1/2	82	16	5·1
Diluted 1/4	35	1	35·0
Diluted 1/8	20	0	—
Sap from tobacco plants inoculated with the Rothamsted culture (TNVb+SV)			
Diluted 1/5	32	403	0·08
Diluted 1/25	19	85	0·22
Diluted 1/125	13	17	0·76
Diluted 1/625	3	1	3·0
Diluted 1/3125	1	0	—

Table 3. *Numbers and ratios of large to small lesions with mixed inocula containing various concentrations of tobacco necrosis virus (TNVb) and constant amounts of satellite virus (SV) at 0·6 mg./l.*

Concentration of large virus (mg./l.)	Size and number of lesions on 8 half-leaves of French bean: Large	Small	Large/small
8·8	53	427	0·12
4·4	47	273	0·17
2·2	22	170	0·13
1·1	7	46	0·15

When a constant amount of TNVa or TNVb was mixed with decreasing amounts of SV, the ratio of large to small lesions in beans increased (Fig. 1). The ratio of large to small lesions also increased when a mixture of TNVb and SV was inoculated at different dilutions (Table 2). Hence the effect on lesion size depends not only on the ratio of the two viruses in the inoculum but also on the absolute concentration of SV; dilution presumably lessens the chances of TNVb and SV particles meeting in the same cell of infection (multiple infection) and interfering with each other. When beans were inoculated with TNVb at different concentrations in a constant concentration of SV, the ratio of large to small lesions remained about the same (Table 3). Fewer lesions were produced by the diluted TNVb; but at a constant concentration of SV the chances that TNVb and SV particles will meet in the same cell are also unchanged.

In all the above experiments TNVb and SV were inoculated simultaneously as

mixtures, but lesion size can be affected when the two are inoculated at different times. When TNVb was inoculated first, however, SV had to be inoculated soon afterwards to affect lesion size. About half the lesions were small when the interval between the two inoculations was 30 min., about one-quarter when the interval was 5 hr. and all lesions were large when the interval was 1 day. By contrast, bean plants inoculated with 40 mg./l. of SV produced only small lesions when re-inoculated with TNVb 5 days later.

Interference in virus multiplication

TNVb multiplies less extensively in leaves inoculated with a mixture of the two viruses than when inoculated alone; the extent to which its multiplication is depressed depends on the ratio of the two viruses in the inoculum. With inocula containing much more SV than TNVb, the amount of TNVb was too small to be detected serologically (Table 4). Other factors than the ratio of the two viruses in the inoculum can influence the virus concentration. SV reached higher concentrations in bean than in tobacco leaves, whereas TNVb inoculated alone reached higher concentrations in tobacco than in bean (Table 5). As SV seems completely dependent on TNVb it is interesting that it multiplies better in the species of plant less well suited for the multiplication of TNVb. In both bean and tobacco, TNVb was more effective than TNVa in stimulating the multiplication of SV.

Table 4. *Concentration of tobacco necrosis virus (TNVb) and satellite virus (SV) in single and mixed infections in tobacco plants, 6 days after inoculation*

	Reciprocals of precipitation end point	
Inocula* (mg./l.)	TNVb	SV
TNVb 9	16	0
TNVb 9+SV 3	8	32
TNVb 9+SV 9	2	16
TNVb 9+SV 27	0	16

* 'Celite' was added in the inocula.

Table 5. *Comparison of the concentration of the two tobacco necrosis viruses (TNVa and TNVb) when inoculated alone or together with SV in bean and tobacco plants*

	Reciprocals of precipitation end-point			
	Bean		Tobacco	
Inocula	TVNa or b	SV	TNVa or b	SV
TNVb*	4	0	16	0
TNVb+SV	0	128	1	16
TNVa	2	0	8	0
TNVa+SV	0	32	0	4

* TNVb and TNVa were at 12 mg./l. and SV at 31 mg./l. The tobacco plants were inoculated with the aid of 'Celite'.

Concentrations of TNVb and SV were affected differently by changing temperature. Although TNVb alone and SV (in plants with both viruses) reached maximum concentration in tobacco plants kept at about 20°, the concentration of SV de-

creased relatively more than that of TNVb when the temperature was raised to 26°. The multiplication of TNVb was inhibited by SV more at lower than at higher temperatures, which agreed with the effects of temperature on the type of lesion. At 26° all the lesions formed by mixed inocula were large (Table 1).

The multiplication of the two viruses depended on whether they were inoculated simultaneously or not. When SV was inoculated together with, or 1 day before, TNVb, SV multiplied more than when inoculated after TNVb. The multiplication of TNVb was decreased more by SV when the two viruses were inoculated together than when TNVb was inoculated 1 day before SV. When TNVb was inoculated one day before SV all the lesions were large, but SV nevertheless multiplied under these conditions (Table 6).

Table 6. *Concentration of tobacco necrosis virus (TNVb) and satellite virus (SV) inoculated at different times to tobacco plants*

First inoculation	Second inoculation	Reciprocal of precipitation end-point	
		TNVb	SV
3 Jan. TNVb*	4 Jan. SV	16	8
3 Jan. TNVb	4 Jan. Water	32	0
2 Jan. SV	3 Jan. TNVb	8	32
2 Jan. Water	3 Jan. TNVb	8	0
3 Jan. TNVb+SV	—	2	32

* TNVb and SV were used at 10 mg./l.; 'Celite' was added in all the inocula.

Properties of SV

Purification. Most of the TNVb present in purified preparations of the mixed Rothamsted culture could be precipitated by ammonium sulphate at 10 to 15 % saturation leaving about 75 % of SV in the supernatant fluid, as shown by serological and infectivity tests. The average numbers of lesions per leaf were 61, 89 and 5, respectively, with the mixture, with the precipitate (containing most of the TNVb), and with the supernatant fluid (containing most of the SV). To remove the remaining TNVb the preparations of SV were centrifuged in sucrose gradients. Purified SV inhibited the lesions formed by TNVb at concentrations as low as 1 μg./l. Concentrations less than 3 mg./l. were not precipitated by homologous antiserum.

Purified preparations of SV scattered light much less than preparations of the same concentration of TNVa or TNVb, and when centrifuged at 75,000 *g* for 3 hr. they formed crystalline pellets. Pellets freshly suspended in water were turbid and contained many crystals of different sizes, most of which dissolved in 24 hr. at 4°. Under the microscope the crystals appeared as rhombic plates, and electron micrograph measurements showed them to consist of particles the size of SV (Pl. 1, fig. 1). The rhombic plates resembled the small crystals photographed by Bawden & Pirie (1945, Fig. 3) from concentrated purified preparations of the Rothamsted culture left for some weeks at 0°.

A preparation of SV twice separated in sucrose gradient columns contained 2·0 % phosphorus, in agreement with the results of Bawden & Pirie (1942) who found the phosphorus content of all tobacco necrosis virus isolates to be between 1·7

and 2·0 %. The particle of SV is one-third to one-half that of TNVb and therefore contains correspondingly less nucleic acid; this has been suggested as a possible reason for its apparent inability to multiply unaided (Kassanis & Nixon, 1961). Since then, brome grass mosaic virus has been reported to contain the smallest infective nucleic acid, with a molecular weight of 1×10^6 (Bockstahler & Kaesberg, 1961). The equivalent molecular weight of SV calculated from the sedimentation constant (50 S) is $1{\cdot}85 \times 10^6$ (Bawden & Pirie, 1945), and of its nucleic acid $0{\cdot}37 \times 10^6$, which is much smaller than the brome grass mosaic virus.

Particle size. Measurements of the diameter of SV particles were from 14·3 to 21·2 mμ according to the technique used for mounting the preparations (Kassanis & Nixon, 1961). Because of the uncertainty about the true size of SV, rows of particles were measured on several electron micrographs of the carbon copies of shadowed crystals of SV. The result depended on which direction in the crystal the measurement was made; one way gave the diameter as 14·4 and the other 16·7 mμ. With a diameter of 16·7 mμ and 1·35 density (the density of bushy stunt virus) the particles would have a weight equivalent to a molecular weight of $1{\cdot}9 \times 10^6$, which is close to that calculated from sedimentation constant (Bawden & Pirie, 1945). Hence 16·7 mμ is possibly the actual diameter of SV.

Table 7. *Inactivation of satellite virus by heat*

20 mg./l. of virus in 0·5 ml. lots were heated for 10 min. at different temperatures and mixed with equal volumes of tobacco necrosis virus (TNVb) at 1·2 mg./l.

Treatment	Size and number of lesions on 8 French bean half-leaves		Survival (%)
	Large lesions	Small lesions	
Unheated	0	170	—
Heated at 70°	21	128	8
80°	47	114	4
85°	95	169	2
90°	195	25	1
95°	199	0	0

Inactivation by heat. SV was heated for 10 min. at different temperatures. After heating it was mixed with constant amounts of TNVb, inoculated to beans, and the large and small lesions counted separately. A few small lesions were formed with SV heated at 90° but none after heating at 95° (Table 7). Serological tests on the sap from inoculated leaves confirmed that SV heated at 95° did not multiply. Heating for 10 min. at 70° destroyed over 90 % of the infectivity, whereas heating at 90° did not affect the ability to precipitate with antiserum. Loss of infectivity without obvious change of the precipitation titre is a feature of many spherical viruses. TNVb lost infectivity in 10 min. between 75° and 80°, whether heated alone or with SV.

Stability. The activity of purified preparations of SV stored at 3° remained unaltered for several months. That some activity remained after 17 years was shown by tests on several purified preparations of the Rothamsted culture of TNV given to me by Mr N. W. Pirie. These had been left at 3° since 1943, and produced no lesions on bean but when mixed (at 50 mg./l.) with active TNVb they decreased the size of

25 % of the lesions. A 17-year-old preparation of the 'potato' isolate of TNV contaminated with the Rothamsted culture of TNV (Bawden & Pirie, 1942) was also inoculated to beans; it produced a few lesions and virus from these was multiplied in tobacco. Partially purified virus prepared from the tobacco contained mostly large particles but also some small ones. The presence of SV suggests that the 'potato' isolate, a stable tobacco necrosis virus (Bawden & Pirie, 1942), is another that can activate SV.

Ultraviolet inactivation. TNVb is inactivated 2½ times faster than SV by ultraviolet irradiation at a wavelength of 254 mμ (Fig. 2).

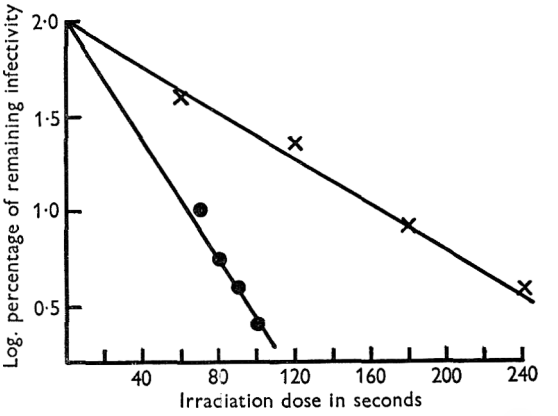


Fig. 2. The inactivation of SV and TNVb (both 20 mg./l.) by ultraviolet light. 20 cm. from source in films 0·07 cm deep. ×—×, SV; ●—●, TNVb.

Ultraviolet absorption. The ultraviolet absorption spectrum of SV was similar to that of TNVb, with a maximum at 260 mμ and a minimum at 241 mμ. The extinction coefficient for SV was 6·5 (for 1 mg./ml.).

Electrophoretic mobility. In 0·066M-phosphate buffer (pH 7·0) SV was stationary even after 3 hr. at a potential gradient of 9·5 v./cm., whereas TNVb had a mobility of $-7{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ v.$^{-1}$. The difference in mobility between the two viruses was found useful in separating them from purified preparations of the Rothamsted culture. SV remained in the descending arm and after 2½ hr. at a potential gradient of 9·5 v./cm., TNVb moved away and the arm was disconnected. Preparations of the Rothamsted culture usually have much less TNVb than SV and electrophoresis gave clean preparations of SV. The two viruses were also separated from a preparation of the Rothamsted culture enriched by adding TNVb so that the mixture contained per weight more of TNVb than SV. SV prepared from this run was almost free from TNVb particles (Table 8). Preparations of SV obtained by electrophoresis from ordinary preparations of the Rothamsted culture were as free from TNVb as those obtained by centrifugation in sucrose gradient columns. Separation by electrophoresis was preferred because it was less wasteful, and its efficiency was not impaired by high virus concentration or by aggregated SV in the mixture. Purified preparations of the Rothamsted culture containing aggregated SV gave three zones when centrifuged in sucrose gradients; the lowest zone was difficult to remove from the tube through the middle zone containing TNVb (Kassanis & Nixon, 1961).

Nucleic acid of SV. When preparations of SV were disrupted with phenol (Geirer & Schramm, 1956) they were still infective and still gave many of the phenomena shown by intact virus. Such disrupted preparations were inactivated in less than a day at 20°. They presumably consisted largely of nucleic acid, for they were rapidly inactivated by dilute pancreatic ribonuclease, which does not inactivate intact virus. Fresh preparations of nucleic acid did not produce lesions when inoculated alone to bean or tobacco and no virus was later recoverable from such inoculated plants. When inoculated together with TNVb, however, these nucleic

Table 8. *Separation of satellite virus (SV) from a mixture with TNVb by electrophoresis*

Inoculum	Average particle count		TNVb/SV	Infectivity test Amount of virus (mg./l.)	
	TNVb	SV		300	30
				Average number of lesions per leaf	
From descending arm	1	435	0·002	2	0
From ascending arm	134	33	4·0	65	17
The mixture before electrophoresis	64	111	0·57	37	14

acid preparations decreased the size of the lesions and such inoculated plants later contained normal SV. To influence the size of lesions produced by TNVb, the nucleic acid and TNVb had to be inoculated simultaneously or the TNVb within an hour or so of the nucleic acid. This is a striking difference from the intact virus which produces its effects when inoculated several days before the TNVb. The difference between the behaviour of the nucleic acid and intact virus presumably largely reflects the greater instability of the nucleic acid. There was, however, some result from an early inoculation with nucleic acid, for although the lesions were all large some SV could later be extracted from such plants. In two experiments out of five, when TNVb was inoculated as long as 2 days after nucleic acid, sap extracted 6 days later contained some SV, although only about 6 % of the amount from leaves inoculated with TNVb immediately after nucleic acid. In the other three experiments of this kind, SV was not detected when the inoculation with TNVb was delayed for 2 days. Different glasshouse temperatures may have caused the inconsistency. Since the nucleic acid preparations lost their infectivity within a day at 20°, it seemed that *in vivo* some nucleic acid was either protected from inactivation by cellular components, or had produced an undetectable amount of new virus at some sites, where multiplication reached detectable amounts after infection with TNVb. TNVb when inoculated with the nucleic acid of SV, as with whole SV, sometimes produced twice as many lesions as when inoculated on its own.

DISCUSSION

The interactions between TNVb and SV seem to differ from the various types already described. There is nothing novel in the multiplication of TNVb being decreased by SV, except that such interference is usually between related strains, whereas TNVb and SV are serologically unrelated. However, tobacco severe etch

virus can suppress the multiplication of potato virus Y, which is not related to it (Bawden & Kassanis, 1945). Nor is there anything new in the idea that one virus may increase the amount to which another multiplies; but the previous examples have all been with viruses clearly able to multiply on their own (Rochow & Ross, 1955; Kassanis, 1961). The striking feature of the interaction between TNVb and SV is that SV apparently depends completely on TNVb for its ability to multiply. However, it is necessary to say apparently, for although when inoculated alone to plants it is not pathogenic and it does not multiply detectably, it is necessary to consider the limitations of the techniques used in detection. The most sensitive technique is the ability to affect the size of lesions produced by TNVb and this can be done by preparations containing as little as 1 μg./l. Serological tests are very much less sensitive than infectivity tests: the minimum amount detectable is 3 mg./l. Obviously, the SV could multiply many times before it became detectable serologically and even by infectivity tests. Attempts were made to demonstrate multiplication in leaves inoculated with SV alone by extracting the sap 10 days later and mixing it with TNVb and inoculating to beans, but all the lesions were large.

There are three reasons for suspecting that SV induces changes in the plant cells and so withstands inactivation, or perhaps multiplies to a limited extent. First, plants inoculated many days earlier with SV still contain considerable amounts of SV when later re-inoculated with TNVb. This could be explained simply by particles from the initial inoculum surviving intact for days in the inoculated leaves until their replication is set going by TNVb. However, the idea conflicts with the behaviour of other viruses as stable as SV, which are inactivated within a few days of being inoculated in hosts where they do not multiply. Secondly, under certain conditions, the infectivity of TNVb is greater when inoculated with SV or its nucleic acid, than when inoculated on its own; some particles of TNVb which otherwise could not have infected can do so in the presence of SV. This suggests that SV changes cells so they become more susceptible to TNVb. Thirdly, and perhaps more important, is the fact that some SV can be recovered from plants inoculated with SV nucleic acid 2 days before they are inoculated with TNVb, for it seems highly improbable that the fragile nucleic acid could survive unharmed for 2 days while waiting to be activated by TNVb. It may be protected from inactivation by association with some host component, perhaps by entering a state comparable to that of bacteriophage in lysogenic bacteria, or it may have initiated virus multiplication which has proceeded only to an undetectable degree.

These are possibilities, but the main picture from the results is one of SV being completely dependent on an attendant virus to create conditions in which it can multiply. The attendant virus seems to lose from the association, because it multiplies less in doubly-infected leaves than on its own. The one suggestion of gain is that occasionally more infection occurs with inocula of TNVb + SV than with inocula of TNVb alone. However, even when this happens most of the lesions are smaller than they would be with TNVb alone and at each infection site its multiplication is restricted.

I am indebted to Dr A. Kleckzkowski for the electrophoresis and Mr R. D. Woods for the electron microscopy.

REFERENCES

BAWDEN, F. C. & KASSANIS, B. (1945). The suppression of one plant virus by another. *Ann. appl. Biol.* **32**, 52.

BAWDEN, F. C. & PIRIE, N. W. (1942). A preliminary description of preparations of some of the viruses causing tobacco necrosis. *Brit. J. exp. Path.* **23**, 314.

BAWDEN, F. C. & PIRIE, N. W. (1945). Further studies on the purification and properties of a virus causing tobacco necrosis. *Brit. J. exp. Path.* **26**, 277.

BAWDEN, F. C. & PIRIE, N. W. (1950). Some factors affecting the activation of virus preparations made from tobacco leaves infected with a tobacco necrosis virus. *J. gen. Microbiol.* **4**, 464.

BOCKSTAHLER, L. E. & KAESBERG, P. (1961). Bromegrass mosaic virus: a virus containing an unusually small ribonucleic acid. *Nature, Lond.* **190**, 192.

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

KASSANIS, B. (1952). Some effects of high temperature on the susceptibility of plants to infection with viruses. *Ann. appl. Biol.* **39**, 358.

KASSANIS, B. (1961). The transmission of potato aucuba mosaic virus by aphids from plants also infected by potato viruses A or Y. *Virology*, **13**, 93.

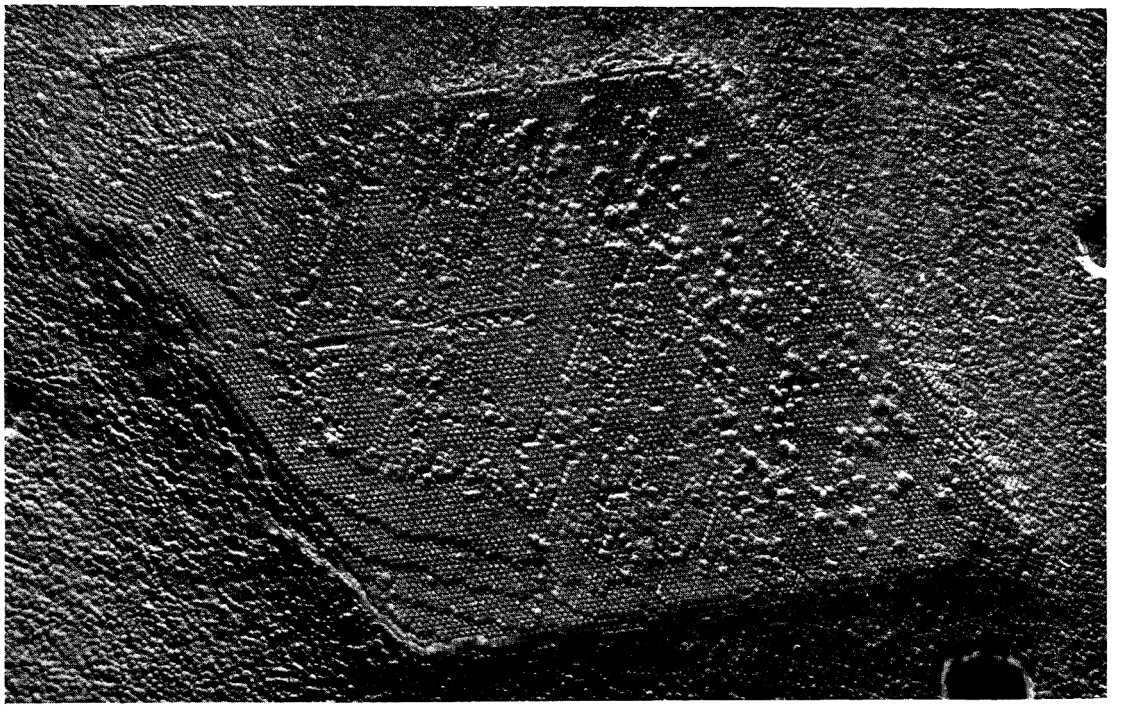
KASSANIS, B. & NIXON, H. L. (1960). Activation of one plant virus by another. *Nature, Lond.* **187**, 713.

KASSANIS, B. & NIXON, H. L. (1961). Activation of one tobacco necrosis virus by another. *J. gen. Microbiol.* **25**, 459.

ROCHOW, W. F. & ROSS, A. F. (1955). Virus multiplication in plants doubly infected with potato viruses X and Y. *Virology*, **1**, 10.

EXPLANATION OF PLATE

Crystal of staellite virus (platinum shadowed carbon replica). × 54,000.



B. KASSANIS

(*Facing p.* 488)

J. gen. Microbiol. (1962), 27, 489–500
Printed in Great Britain

A Photometric Method for Following Changes in Length of Bacteria

By E. O. POWELL and P. J. STOWARD*
The Microbiological Research Establishment, Porton Down, Salisbury, Wiltshire

(*Received* 19 *July* 1961)

SUMMARY

Rod-shaped particles suspended in a liquid undergo a partial orientation during flow; the flowing suspension has the property of scattering light mainly in certain preferred directions. The elementary theory of the phenomenon shows that it can be applied practically as a photometric method of following changes in length. A simple and effective apparatus for this purpose is described, and its performance illustrated. A brief discussion of size changes in synchronized cultures indicates the relation between these changes and the rate of fission.

INTRODUCTION

Progress in the study of synchrony in bacterial cultures has been handicapped in the past by the absence of a rapid and convenient means of measuring the concentration (by number) of a suspension. Conventional total or viable counts are slow and tedious, and do not meet the need for an immediate check on the occurrence of synchronous fission, and so on the efficacy of a trial procedure for inducing synchrony. Even a quite rough measurement would be acceptable, as indicating the occasions on which it would be profitable to carry out more exact counts. Instruments like the 'Coulter Counter', which enumerates the number of particles in a minute volume of suspension, have not yet been developed to the point at which they are both reliable and easy to use with bacterial suspensions.

As a method of monitoring if not of actual estimation, some form of photometric measurement would be especially convenient, but it is not to be expected that any standard instrument would be directly applicable. The turbidity of a suspension depends radically on both the size and concentration of the particles in it (other things being equal), and its true optical density bears no simple relation to these variables. In addition any conventional instrument intended as an absorptiometer gives, when used with suspensions, estimates of an apparent optical density which differs grossly from the true one and which is a function of the geometry of the instrument (for examples, see Powell, 1962). It is often supposed that turbidity measurements correspond with concentrations of organisms by mass, and this is roughly and empirically true of certain combinations of instrument and suspension, but there are many recorded examples to the contrary, in particular the interesting experiment of Stárka & Koza (1959) discussed below.

In well-developed synchronous growth, there is no indication of any large fluctuation in the mass growth rate, but during periods of fission, the number concentra-

* Present address: Department of Human Anatomy, South Parks Road, Oxford.

tion is nearly doubled and the mean length of the organisms is nearly halved. These two changes compensate one another to some extent so far as the light scattering of bulk suspensions is concerned. While it is possible in principle to determine the size and shape of suspended particles from their scattering properties, the measurements required are somewhat elaborate in the most nearly ideal cases. Bacteria, differing in size and shape among themselves and not optically homogeneous, are quite intractable theoretically.

In this paper we describe a simple form of photometric measurement which gives a direct indication of the axial ratio of rod-shaped particles and is almost independent of their concentration by number. It is thus particularly suitable for detecting the change in length which occurs during periods of fission in a synchronized culture and for this purpose no calibration is really necessary. The method is based on two phenomena: (i) non-spherical particles scatter light in certain preferred directions which depend on their attitude relative to the direction of the incident light; (ii) in the laminar flow of a suspension, non-spherical particles tend to take up certain preferred orientations wherever there is a velocity gradient normal to the direction of flow. These phenomena are manifested in the familiar nacreous appearance of stirred suspensions of rod-shaped organisms, and in the fluctuating readings given by absorptiometers with such suspensions when they have not come to rest.

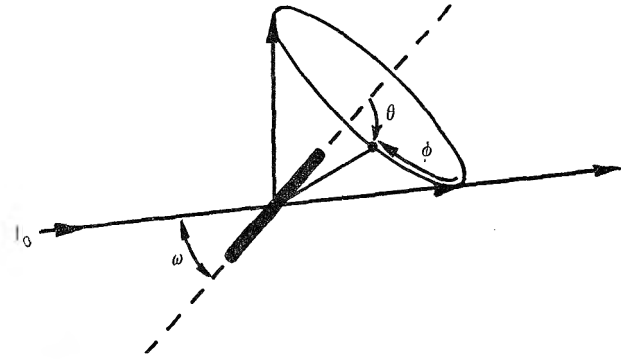


Fig. 1. Preferred scatter by a rod-shaped particle. The rod makes an angle ω with the incident beam I_0. The scattered intensity varies with the azimuth ϕ about the rod as axis, but for a given ϕ it is a maximum for colatitude $\theta = \omega$.

Theory of the method

Since bacteria are of the same order of size as the wavelength of visible light, the light scattered by a suspension is for the most part deflected little out of the direction of the illuminating beam. Whatever the shape of the particles, the scatter from an appreciable volume of suspension is axially symmetrical about the beam when the suspension is stationary, because the particles are oriented at random, but the scatter from a single rod-shaped organism is strongly dependent on direction.

The simple theory of scattering by a long and very thin rod has been known and applied for many years (Siedentopf, 1912; Stadie, 1928). The important features are shown in Fig. 1; the scattered light is concentrated in a cone whose axis is that of the rod and one of whose generators is the direction of the incident beam. When the rod is normal to the beam, the cone opens out into a flat sheet. The behaviour

of rods which are neither very thin nor very long (relative to the wavelength) is qualitatively similar, but the scattered light is less completely concentrated in the preferred directions; the degree of concentration is a measure of the length:thickness ratio. Hence, if we have a means of aligning the particles in a suspension so that their long axes are normal both to an incident light beam and to a convenient direction of observation, the intensity of the scattered light will be greater than if the particles were oriented at random, and the excess will be a measure of the axial ratio.

Partial alignment is readily achieved by allowing the suspension to flow through a tube; the particles tend to set their long axes parallel to the length of the tube. The hydrodynamic forces acting on the particles have to compete with the disorganizing effect of thermal motion (depending on the viscosity of the medium, the temperature, and the moment of inertia of the particles). The alignment is best near the wall of the tube, where the velocity gradient is highest; on the axis there is no alignment since there is no velocity gradient. In spite of this defect, flow through a cylindrical tube is so simple to arrange that it is much to be preferred to any more nearly ideal alternative.

An estimate of the flow rate necessary to effect a useful degree of orientation can be obtained in the following way.

We may expect the orientation to be effective if the shear is large compared with the rotational Brownian movement of the particles (expressed as mean square angular displacement per unit time). The rotational Brownian movement is given by

$$\frac{\overline{\omega^2}}{t} = \frac{2kTP}{3\eta V},$$

where ω is the angular displacement from a given direction, t is time, k is Boltzmann's constant, T is absolute temperature, η is the viscosity of the fluid, V is the volume of the particles, and P is a shape factor (implicitly involving the radius of gyration of the particles). For spheres, P is 1, and for long rods it takes smaller values (Sadron, 1953); we shall not underestimate the rotation if we take $P = 1$. Then very roughly

$$\begin{aligned} k &= 1{\cdot}4 \times 10^{-16} \text{ ergs/degree Centigrade.} \\ T &= 300^\circ \text{ absolute,} \\ \eta &= 0{\cdot}01 \text{ poise (water),} \\ V &= 10^{-12} \text{ ml. (bacteria),} \end{aligned}$$

whence

$$\overline{\omega^2}/t = 2{\cdot}8 \text{ sec.}^{-1}.$$

Now in the laminar flow of a liquid through a cylindrical tube of diameter d, the velocity gradient at the wall is

$$\frac{32Q}{\pi d^3} \text{ sec.}^{-1},$$

where Q is the volume flow rate. We therefore need to make

$$\frac{Q}{d^3} \gg \frac{2{\cdot}8\pi}{32} \doteq 0{\cdot}27.$$

If we work with a tube of diameter 0·5 cm., Q should be much greater than 0·04 ml./

sec. This is a very modest requirement; we can expect good orientation over most of the cross-section with a flow of about 1 ml./sec. In these circumstances the light scattered by the flowing suspension will not be unduly sensitive to changes in flow-rate, which incidentally is much below the critical value for the onset of turbulence.

Both the Brownian motion and the flow through the tube are inversely proportional to the viscosity of the suspending fluid. Changes in viscosity due to temperature variation will therefore not affect the degree of orientation. The additional direct dependence of Brownian motion on absolute temperature will give rise to a small effect, probably negligible for ordinary fluctuations in ambient temperature.

The problem of scatter by a suspension undergoing shear orientation can be treated theoretically if the particles are very thin (Wippler, 1954); it is again an empirical fact that qualitatively similar behaviour is exhibited by bacteria, in spite of their less amenable shape and size.

METHOD

The indications of the preceding section were readily realized by a simple modification to a standard Pulfrich turbidimeter. In this instrument, as normally used, the suspension under test is contained in a cylindrical cuvette immersed in a water-jacket. (The jacket prevents appreciable refraction of the light entering and leaving the cuvette and helps to suppress reflexions.) Light scattered by the suspension over a range of angles near 45° to the illuminating beam is collected by a lens and compared with the lamp intensity in a prismatic eyepiece.

The drain-cock of the water-jacket was removed and a glass tube, about 5 mm. internal diameter and 15 cm. long, was mounted centrally in the chamber. To its lower end was fused a capillary 0·5 mm. internal diameter and 7 cm. long, to its upper end a three-way tap communicating with the open air and with a water-pump (Fig. 2). The tube could thus be filled from below by suction and the contents allowed to drain out under gravity. The drainage rate was about 0·4 ml./sec.—a higher rate would have been preferable but for the restricted sample volume.

The volume of sample required to fill the tube was about 5 ml.; a larger volume did not seem desirable. During draining, therefore, steady flow past the illuminating beam was maintained for only a few seconds, and this was too short a time to permit visual comparison of the scattered light with the illuminant. The eyepiece was therefore replaced by two photoelectric cells working into a symmetrical current amplifier (Fig. 3). The amplifier was based on a reliable circuit due to Sowerby (1944), giving in this case a gain of about 10^6. Sowerby described this circuit incidentally as part of an optical instrument, and its great virtues have in consequence gone largely unrecognized. It can often be used where something less than the full sensitivity of a photomultiplier is adequate. Its special feature is simply the use of very large photocell loads together with correspondingly large cathode loads for the input valves, which are of a standard type. The essential parts of the circuit are shown in Fig. 4. Electrical supplies were derived from a conventional transformer and rectifier system with gas discharge voltage stabilizers, connected to mains through a constant voltage transformer with harmonic filter; this transformer also supplied the photometer lamp (Messrs Advance Components, Ltd., Hainault, Essex. The more usual simpler type of transformer giving merely a constant r.m.s. voltage was found to be less effective, possibly because of its poor wave form).

The zero indicator was a 200 μA. meter (M, Fig. 4) with two sensitivity controls, one continuous (R_{12}) and one a four-position switch (S) giving accurately factors of $\times 1$, $\times \frac{2}{5}$, $\times \frac{1}{10}$, $\times \frac{1}{25}$. The shunt and series resistors associated with the switch were carefully adjusted so that changing the switch position did not alter the resistance presented across the cathodes of V_5.

The fine and coarse adjustments P_1, P_2 (Fig. 4) provide for balancing the amplifier so that the meter reading is zero with no light incident on either photocell.

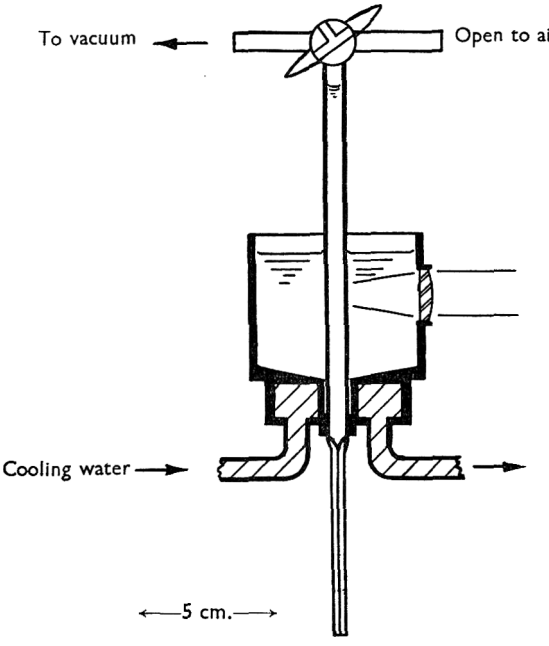


Fig. 2. Sample tube mounted in chamber of standard Pulfrich photometer (sectional elevation). Lenses for admission of light and observation are mounted in the chamber wall. Cooling water may be passed through a separate duct in the base.

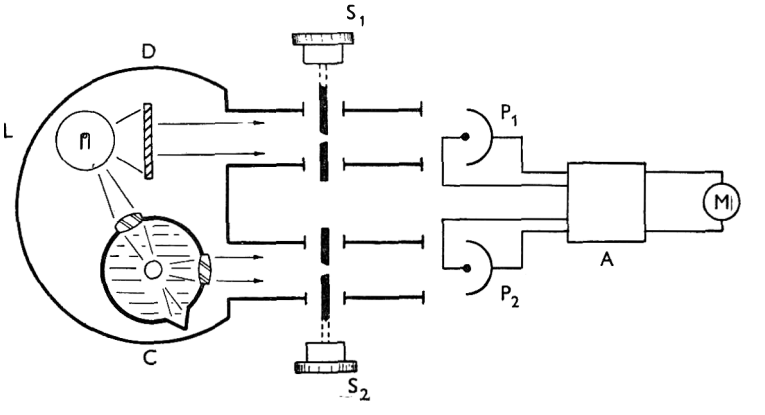


Fig. 3. Modified Pulfrich photometer, plan (schematic). The sample tube is seen in transverse section at the centre of the chamber C. L, lamp; D, diffusing screen; S_1, S_2, calibrated stops; P_1, P_2, photocells; A, amplifier; M, microammeter.

In using the apparatus it was found very necessary to guard against incrustations and bubbles attached to the inner wall of the tube. As a washing liquid and for blank determinations we used normal saline with small additions of phosphate, formalin and an anionic detergent (Norris & Powell, 1961). The detergent promoted foaming, but the bubbles formed did not adhere to the wall. Whenever possible, i.e. when samples of a growing culture had not to be kept at constant temperature, the formation of bubbles of dissolved gas was discouraged by cooling the chamber slightly with tap-water (Fig. 2). As usual in turbidimetric work, care had to be taken to exclude foreign particles, especially cotton wool; washing and diluting fluids were filtered twice through the same paper before use.

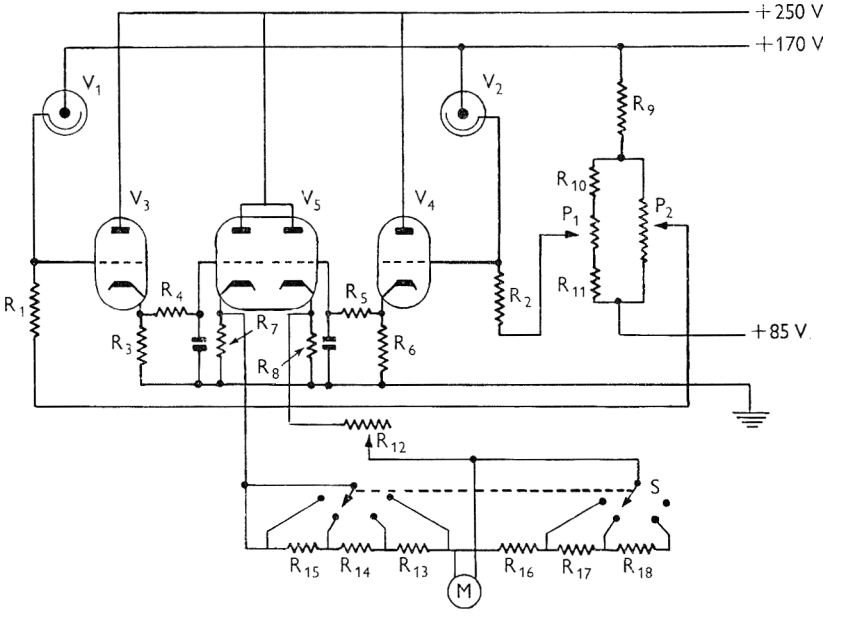


Fig. 4. Circuit diagram of amplifier, omitting power supplies. V_1, V_2, photocells (Cinema-television type VA17T); V_3, V_4, type 6Q7 (diodes strapped to earth); V_5, type 12AT7; R_1, R_2, 200 MΩ; R_3, R_4, R_5, R_6, 1 MΩ; R_7, R_8, 15 kΩ; R_9, 68 kΩ; R_{10}, R_{11}, 10 kΩ; R_{12}, 2·5 kΩ variable; P_1, 2 kΩ potentiometer; P_2, 20 kΩ potentiometer; C_1, C_2, 0·1 μF.; R_{13}, 164·0 Ω; R_{14}, 82·0 Ω; R_{15}, 16·0 Ω; R_{16}, 11·4 Ω; R_{17}, 18·9 Ω; R_{18}, 152·0 Ω; M, 200 μA., 270 Ω; S, 2-pole, 4-way switch.

The apparatus is used in the following way.

A blank determination of parasitic scattered light is first made by filling the sample tube with saline. The calibrated stop S_2 (Fig. 3) is opened to its fullest extent. Then the stop S_1 is adjusted to give the null point on the meter M. The reading (B) of S_1 is recorded. The saline is sucked out; the tube is washed through three times with small quantities (*c.* 0·5 ml.) of the suspension to be tested, and then filled slowly with it (to avoid bubble formation through too much reduction of pressure). By means of the sensitivity controls (R_{12}, S, Fig. 4) the meter reading is brought to exactly 100 μA.; suspensions of long organisms give a constant reading only after 1–2 min. at rest. The reading then corresponds to the light intensity scattered by the suspension itself. The meter is brought to zero again by opening S_1; the new reading (T) of S_1 is noted. (The difference $T-B$ gives a useful comparative

measure of concentration for suspensions of similar organisms.) The three-way tap is then turned to admit air above the suspension; as it drains out, the meter reading rises within 1 or 2 sec. to a maximum value. The foregoing procedure provides that the maximum reading in microamperes is the percentage increase in the scattered light caused by shear orientation. If the increase is small, the operation is repeated, and before the suspension is allowed to drain, the meter sensitivity is increased to a higher value with the switch S (Fig. 4). The new maximum reading then exceeds the true increase by an exactly known factor. In any case it is desirable to repeat the observation two or three times. A defect of the apparatus is the change in flow-rate as the liquid level falls. Fairly reproducible readings are obtained by working only with the meniscus between roughly fixed limits, e.g. the uppermost 3–4 cm. of the tube.

To avoid mechanical damage, the meter sensitivity is set to its extreme minimum (both controls) during emptying and filling of the sample tube.

Some representative results

This apparatus has been used successfully in collaboration with Dr J. R. Postgate for following the growth of synchronized cultures, but our purpose here is to give no more than an indication of its scope and possibilities.

Table 1. *Examples of increased light scattering by flowing suspensions in the modified Pulfrich turbidimeter*

	Shear effect (% increase)
Polystyrene spheres (1·8 μ diam.)	0
Chromobacterium prodigiosum (very small resting cells)	5
Aerobacter aerogenes (from steady growth in continuous culture)	28
Human erythrocytes in saline	12

A general impression of the magnitudes involved is given by Table 1. Human erythrocytes are included as an example to show that the application can be extended to non-spherical particles other than rods; in a number of industries there are control problems to which the principle might be applied: cosmetics, paint, explosives. For convenience, we use the phrase 'shear effect' to mean the percentage increase in light scatter during flow, as measured in the apparatus we have described.

If the concentration and thickness (in the direction of the illuminating beam) of a sample of a suspension are not too large, the individual particles scatter independently of one another and their effects are additive. Van de Hulst (1957) suggests as a working rule that this is true up to an optical density of about 0·1. Within this range we should expect the shearing effect (which is a ratio of turbidities) to be independent of concentration, and this is in fact nearly true up to appreciably higher densities. The useful concentration range of the apparatus we have described is, for bacteria, from about 2×10^7 to 2×10^9 per ml., depending on the kind of organisms, but for synchrony work the concentration chosen should be as low as is convenient. The shearing effect is then influenced by changes in shape but not in number. Table 2 illustrates the dependence of shear effect on concentration.

The shear effect is not greatly influenced by the colour of the light. At high concentrations violet light produces a detectably smaller effect than green or red (presumably because the optical density is higher for the shorter wavelength), but there seems to be no reason for using filtered light in ordinary practice.

Table 2. *Dependence of shear effect on concentration.* Aerobacter aerogenes *in saline. Mean length of organisms:* 5·47μ; *mean breadth:* 0·50μ; *ratio,* R, 10·8

Concentration, No./ml. $\times 10^{-9}$	Turbidity (arbitrary units)	Shear effect (% increase)
2·2	50·9	19·6
0·75	20·8	26·5
0·25	6·1	33·9
0·083	1·9	35·9

Suspensions of bacteria usually contain organisms having a considerable dispersion of size, and the shearing effect observed is a weighted mean of the various effects due to the individual particles. Suppose we write T_r for the scattered intensity from a given suspension at rest and T_s for the scattered intensity from the flowing suspension, then what we have called the shear effect is

$$100(T_s/T_r - 1).$$

It is possible to write down a formal expression for T_s/T_r as a weighted mean of contributions from the different particles, but it is of little practical value because the measured scatter depends in a very complex way on both general optical laws and on the geometry of the apparatus. However, the way in which shear effects combine can readily be illustrated. Suppose we have two suspensions, a and b, giving scattered intensities $T_{r,a}$, $T_{r,b}$ (stationary) and $T_{s,a}$, $T_{s,b}$ (flowing); then in a mixture with volume fractions α and β of a and b, respectively, we have

$$T_r = \alpha T_{r,a} + \beta T_{r,b},$$
$$T_s = \alpha T_{s,a} + \beta T_{s,b},$$

provided the concentrations are low enough to be directly proportional to turbidity in each suspension severally. Then

$$\frac{T_s}{T_r} = \frac{\alpha T_{r,a}}{\alpha T_{r,a} + \beta T_{r,b}} \frac{T_{s,a}}{T_{r,a}} + \frac{\beta T_{r,b}}{\alpha T_{r,a} + \beta T_{r,b}} \frac{T_{s,b}}{T_{r,}};$$

writing C, C_a, C_b for the shear effects (so that $C = 100\,(T_s/T_r - 1)$, etc.),

$$C = \frac{\alpha T_{r,a}}{\alpha T_{r,a} + \beta T_{r,b}} C_a + \frac{\beta T_{r,b}}{\alpha T_{r,a} + \beta T_{r,b}} C_b.$$

The truth of this equation was tested in a number of experiments by making up mixtures of two radically different suspensions, and comparing the observed and calculated shear effects. The agreement was uniformly satisfactory; Fig. 5 gives an extreme example.

Figure 6 is a conspectus of the association between shear effect and the ratio, R, of the mean length to mean breadth of the organisms in the suspensions concerned. It covers a wide variety of suspensions, including several samples from continuous cultures in which growth restriction had produced exaggerated forms of size-distribution. The correlation is perhaps less perfect than might at first be expected;

the estimates of R were based each on the measurements of rather few organisms—never more than 40—and are subject to appreciable errors, both statistical and experimental. It has in any case to be remembered that suspensions having the same value of R will give different shear effects if their size distributions are different. Moreover, the shear effect is not a function, even in homogeneous suspensions, of the shape only, but depends also on the absolute size and refractive index, and is affected by non-uniformity within the particles (e.g. organisms in a state of incipient fission). Spores of *Bacillus alvei* provide an example; the spores are enclosed in spindle-shaped envelopes with long pointed ends and of low optical density. They are represented in Fig. 6 by two entries; for the one, R is taken to be

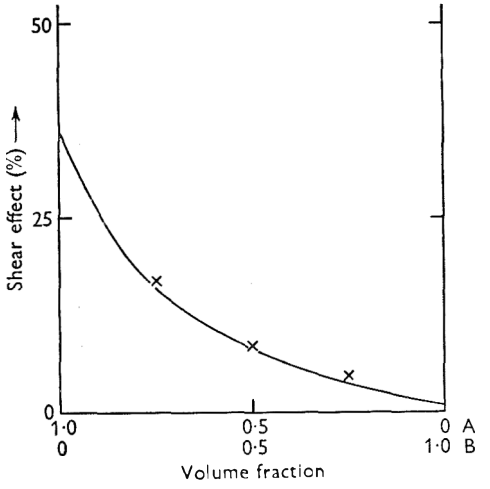


Fig. 5

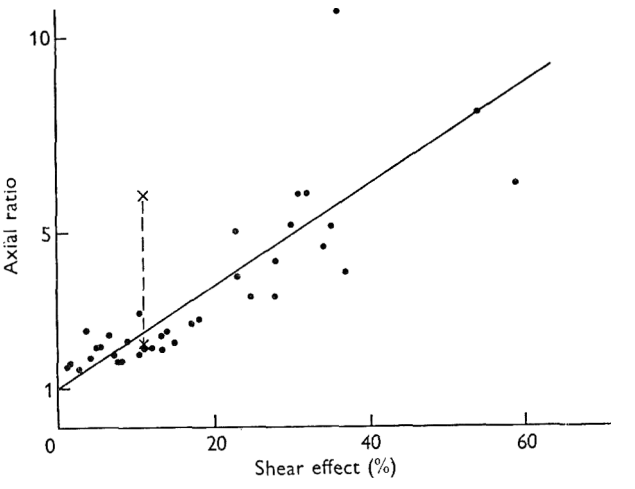


Fig. 6

Fig. 5. Shear effect in mixtures. Suspension A: *Aerobacter aerogenes*, $8{\cdot}3 \times 10^7$/ml.; relative turbidity, 1·7; shear effect 36·0 %. Suspension B: *Bacillus subtilis* spores, $1{\cdot}23 \times 10^8$/ml.; relative turbidity, 6·9; shear effect 1·1 %. Continuous curve, calculated; crosses, experimental values.

Fig. 6. Summary diagram of relation between shear effect and axial ratio; various suspensions. *Bacillus alvei* spores are represented by two entries joined by a dotted line. The larger axial ratio is that of the envelope, the smaller that of the spore itself. The solid line is the regression of axial ratio on shear effect (assumed linear).

the ratio of the extreme dimensions of the spindle, for the other, of the dense central mass only. By the one standard, the shear effect is unusually low; by the other, unusually high.

Size fluctuation in synchronous growth

Stárka & Koza (1959) described an experiment in which the growth of a synchronized culture was followed photometrically as well as by counting the viable organisms. They found that in their apparatus the turbidity increased in the same stepwise manner as the number concentration; during periods of growth without fission the turbidity did not change in spite of the increasing size of the organisms; during periods of rapid fission it followed the increase in number. This rather surprising result is due, we think, to the geometry of the instrument used—the Pulfrich turbidimeter with scattered light collected at 45° to the illuminating beam.

An increase in particle size brings about a greater concentration of the scattered light in directions close to the incident beam, and a reduction in intensity in remote directions; there will be a range of angles within which the intensity changes little. This condition seems to apply here; to some extent Stárka & Koza's result is a happy accident. We have carried out an experiment of the same kind, using a similar turbidimeter and a synchronous culture of *Aerobacter aerogenes* obtained by fractional filtration according to the method of Abbo & Pardee (1960). The stepwise change in turbidity was well-marked though less sharp than in Stárka & Koza's experiment. Similar measurements carried out by Abbo & Pardee with a different form of photometer showed much less clearly marked steps.

In well-developed synchronous growth the number concentration very nearly doubles during the fission phase, and the above example shows that in favourable cases the turbidity may do the same. The changes in mean size which occur, however, are not only smaller but less rapid, as is shown qualitatively by the following hypothetical illustration.

Suppose we have a suspension of organisms all of which have just divided at time $t = 0$, such as might be obtained by fractional filtration of a growing culture. Assume (i) that the organisms all have the same length x_0 initially; (ii) that the diameter of the organism is constant but the length x (hence the volume) increases exponentially at the same rate ν as the steady growth rate of the culture: $x = x_0 e^{\nu t}$; (iii) that the distribution $f(\tau)$ of generation times τ is so narrow that almost all the organisms have divided before any of the first-generation progeny has done so. Then if there are initially N organisms, the actual fission rate dN/dt will be $Nf(t)$ during this period. At time t there will be

$$2N\int_0^t f(\tau)\,d\tau = \text{say } 2NF_+(t),$$

first-generation progeny, of mean length $\frac{1}{2}x_0 e^{\nu t}$, and

$$N\int_t^\infty f(\tau)\,d\tau = N\{1-F_+(t)\},$$

original organisms still undivided, of length $x_0 e^{\nu t}$. The mean length is thus

$$\bar{x} = \frac{N\{1-F_+(t)\}x_0 e^{\nu t} + NF_-(t)\,x_0 e^{\nu t}}{N\{1-F_+(t)\}+2NF_+(t)};$$

$$\frac{\bar{x}}{x_0} = \frac{e^{\nu t}}{1+F_+(t)}.$$

For the purpose of the illustration we may take $f(\tau)$ to be a Pearson Type III distribution

$$f(\tau) \equiv \frac{\tau^{g-1}e^{-g/m}}{m^g\,\Gamma(g)}.$$

The value of the parameter m affects only the time-scale—we may take $m = 1$; g is a measure of the dispersion, and a representative value of 35 is suggested by actual measurements (Powell, 1958). Then $\nu = 0{\cdot}0200$ min.$^{-1}$. Hence $\bar{x}$ may be calculated as a function of t. The result is shown in Fig. 7; in this case the maximum mean length is only $1{\cdot}6x_0$, not $2x_0$; the fall in $\bar{x}$ occurs during the earlier stages of the fission phase and is less striking than the increase in numbers. (This simple reasoning no longer applies after the first-generation progeny have begun to divide.)

In real cultures, there is always an appreciable dispersion of size in the initial population, and it is not known that individual organisms grow at the same constant rate. Nevertheless, the example will serve as a warning not to expect a simple geometrical relation between the curves representing changes in mean size and changes in number. In fact the example agrees quite well with such experimental figures as have been obtained; we are indebted to Dr J. R. Postgate for providing data for Fig. 8.

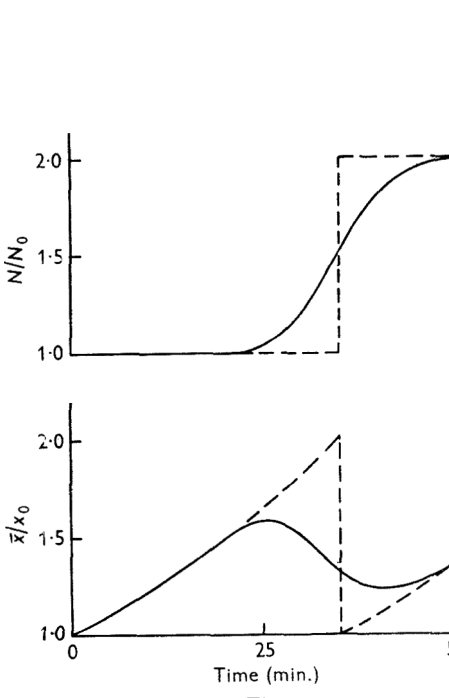


Fig. 7

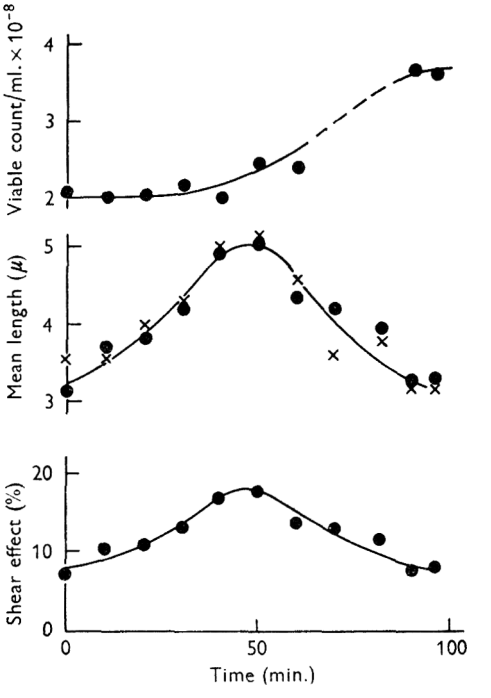


Fig. 8

Fig. 7. Illustrating variation of mean length of organisms in synchronous growth. Upper curves: increase in numbers (N); lower curves: changes in mean length ($\bar{x}$). The dotted curves show the result to be expected in the absence of any dispersion of generation time. Calculated for mean generation time 35 min., standard deviation 5·9 min.

Fig. 8. Early development of a culture of *Aerobacter aerogenes* synchronized by repeated starvation. Upper curve: viable count. Lower curve: shear effect. Middle curve: crosses—measured mean length; circles—mean length estimated from shear effect (J. R. Postgate).

Further development

The apparatus we have described could not well be simpler, but it is in many cases quite adequate for checking the development of supposedly synchronized cultures. Its sensitivity, reproducibility, and range of application could readily be improved by attention to some obvious defects, particularly the poor control of flow-rate and temperature, and the large and imperfectly compensated blank reading due to reflexions at glass-liquid interfaces.

The sensitivity, in particular, could in principle be increased by illuminating the sample with polarized light, but rough experiments suggest that the advantage would be slight. A particular suspension, for example, giving a shear effect of

28·1 % in natural light gave in polarized light values of 26·2 and 30·7 % when the electric vector was respectively perpendicular and parallel to the axis of the sample tube. As we constructed it, the apparatus is not quite accurate enough for work on organisms of low axial ratio such as spores (R = 1·5, e.g.) which give a shear effect of only 1–3 %. A more accurate instrument would make it worth while to investigate in more detail the dependence of shear effect on dispersion of size. It is clear that the arithmetic means we have used are not really appropriate to the problem, and some form of weighted mean might be found, much more closely correlated with the shear effect.

It should also be possible, by a refinement of the electronic detector, to measure the dispersion as well as the mean of an axial ratio. When the flow of a suspension is suddenly stopped, the shear orientation exhibits a relaxation time which is surprisingly long—frequently many seconds elapse before the turbidity reverts to its stationary value (cf. the estimate of rotational Brownian motion on p. 491 above). Consider two suspensions, the first of particles of moderate axial ratio, the second of a mixture of spheres with very long particles, adjusted to have the same shear effect as the first. Then the relaxation time of the second will be the same as if it contained the long rods only, since the spheres contribute nothing to the shear effect. The relaxation time of the homogeneous suspension will be the shorter. Hence if we can estimate the rate of decay of the shear effect (i.e. of the excess scattered light) when the flow is stopped, the result can be used in combination with the magnitude of the effect as a measure of the dispersion of axial ratio.

We are indebted to W. C. Wright for constructing the photoelectric detector, and to P. F. Errington for making many measurements. One of us (P. J. S.) carried out this work while acting as a Vacation Consultant from the College of Advanced Technology, Birmingham.

REFERENCES

ABBO, F. & PARDEE, A. B. (1960). Synthesis of macromolecules in synchronously dividing bacteria. *Biochim. biophys. Acta*, **39**, 478.

VAN DE HULST, H. C. (1957). *Light Scattering by Small Particles*. New York: John Wiley and Sons, Inc.

NORRIS, K. P. & POWELL, E. O. (1961). Improvements in determining total counts of bacteria. *J. R. micr. Soc.* **80**, 107.

POWELL, E. O. (1958). An outline of the pattern of bacterial generation times. *J. gen. Microbiol.* **18**, 382.

POWELL, E. O. (1962). Photometric methods in bacteriology. *J. Sci. Fd Agric.* (In the Press.)

SADRON, C. (1953). Methods of determining the form and dimensions of particles in solution: A critical survey. In *Progress in Biophysics*, **3**, 237. Ed. J. A. V. Butler & J. T. Randall. London: The Pergamon Press, Ltd.

SIEDENTOPF, H. (1912). Über ultramikroskopische Abbildung linearer Objekte. *Z. wiss. Mikr.* **29**, 1.

SOWERBY, J. McG. (1944). A photoelectric photometer for measuring the light scattered by the surface of a transparent material. *J. sci. Instrum.* **21**, 42.

STADIE, F. (1928). Probleme der Brownschen Molekularbewegung. *Ann. Phys. Lpz.* **86**, 751.

STÁRKA, J. & KOZA, J. (1959). Nephelometric determination of cell count in synchronously dividing cultures of bacteria. *Biochim. biophys. Acta*, **32**, 261.

WIPPLER, C. (1954). Étude théorique de la diffusion de la lumière par les sols de batonnets orientés. *J. Chim. phys.* **51**, 122.

J. gen. Microbiol. (1962), 27, 501–508
Printed in Great Britain

Some Properties of the Complement-fixing Antigens of the Agents of Trachoma and Inclusion Blennorrhoea and the Relationship of the Antigens to the Developmental Cycle

By P. REEVE and JANICE TAVERNE
M.R.C. Trachoma Research Unit, The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1

(*Received* 21 *July* 1961)

SUMMARY

The viruses of trachoma and inclusion blennorrhoea, like other members of the psittacosis-lymphogranuloma group, contain heat-stable and heat-labile complement-fixing antigens. The heat-stable antigen, which is soluble in ether and partly destroyed by periodate, appears to be chemically similar, as well as serologically related to, the lipopolysaccharide-protein complex shared by other viruses of the group. Complement-fixing group antigen was detected at all times during one cycle of multiplication of a strain of inclusion blennorrohea virus in HeLa cells. The amount of cell-associated antigen, about half of which sedimented under the same conditions as mature elementary bodies, remained constant for the first 16 hr. after adsorption; after 16 hr. it increased linearly, reaching a maximum 28 hr. after adsorption. The group antigen probably forms at a stage in the developmental cycle when large forms predominate and is later distributed among the smaller infectious elementary bodies.

INTRODUCTION

Bedson (1936) first observed that psittacosis virus possesses both heat-labile and heat-stable complement-fixing antigens. The heat-stable antigen, which is shared by other viruses of the same group (Bedson, Barwell, King & Bishop, 1949; Monsur & Barwell, 1951; Ross & Gogolak, 1957) is partly destroyed by periodate (Barwell, 1952; Sigel & Pollikoff, 1953) is soluble in ether (Hilleman & Nigg, 1946) and is associated with a phosphatide (Volkert & Christensen, 1954). Benedict & O'Brien (1956) found the periodate-sensitive fraction of group antigen extracted from feline pneumonitis with sodium lauryl sulphate to be a lipopolysaccharide, whereas the fraction which resists treatment with periodate is a protein. We have found that the viruses of trachoma and inclusion blennorrhoea are antigenically similar to other members of the group and have investigated the relationship of the lipoprotein-polysaccharide group antigen to infective virus, by following its development during one cycle of multiplication of inclusion blennorrhoea virus in HeLa cells.

METHODS

HeLa cells. Monolayers of HeLa cells were grown in 250 ml. Pyrex feeding bottles. Each bottle contained 10 ml. medium consisting of Hanks's saline solution containing 10 % (v/v) human serum, 5 % (v/v) calf serum, 0·5 % (w/v) lactalbumin hydrolysate and 1,000 units streptomycin/ml.

Viruses. The following strains were used:

Trachoma: TE55 (T'ang, Chang, Huang & Wang, 1957);
LT1 (Jones, 1961).

Inclusion blennorrhoea:
LB1 (Jones, Collier & Smith, 1959);
LB2, LB3 and LB4 (Jones, 1961).

Disruption of HeLa cells. Infected cells were suspended in 5 ml. diluent (see below) and submitted for 4 min. to a frequency of 20 kc./sec. produced by an MSE-Mullard ultrasonic disintegrator with a power output of 60 W. (Furness, Graham & Reeve, 1960).

Diluent. The diluent used consisted of 0·02 M-H_3BO_3, 0·0008 M-$Na_2B_4O_7$, 0·0007 M-$CaCl_2$, 0·003 M-$MgCl_2$ and 0·15 M-NaCl in distilled water, to which was added 2 % (v/v) heated normal guinea-pig serum.

Antigens. Virus grown in yolk sac or HeLa cells was sedimented at 8000 *g* for 40 min. at 4° and resuspended in a volume of diluent giving at least a ten-fold concentration of the original virus suspension. When required, antigens were heated for 30 min. at 100° in a water bath.

Periodate treatment. Equal volumes of antigen and 0·025 M-KIO_4 in 0·15 M-NaCl were mixed and incubated at 37° for 30 min. A drop of the mixture liberated iodine from a drop of 1 % (w/v) KI in 0·2 M-phosphate buffer (pH 7·0), indicating that KIO_4 was in excess, and this excess was then neutralized with 20 % (w/v) glucose.

Antiserum. Rabbits were immunized by intravenous injection of yolk-sac or cell-culture suspensions of virus (Reeve & Graham, 1962). Virus suspensions were administered to guinea pigs intranasally. Sera were stored at −40° and were heated for 30 min. at 56° before use.

Complement. Fresh guinea-pig serum stored at −40° was used.

Complement fixation. Antigens were titrated by the method of Fulton & Dumbell (1949), with a 1/20 dilution of antiserum known to give maximum fixation with concentrated antigen. Reaction mixtures were left at 4° overnight before the haemolytic system was added, since this more than doubled the amount of complement fixed. Dilutions of antigen were plotted against $\log_{10}$ units of complement fixed; the antigen titre was taken as the reciprocal of the dilution fixing 1 unit of complement.

Growth cycle experiments. HeLa cell monolayers were inoculated with 2 ml. of a suspension of virus, obtained from ultrasonically disrupted cells, at a concentration known from routine infectivity titrations to infect every cell (Furness *et al.* 1960). Virus was allowed to adsorb for 4–6 hr. at 30°; unadsorbed virus was then decanted, the monolayers washed twice with 10 ml. 0·15 M-NaCl, then 10 ml. tissue culture medium added and the cultures incubated at 37°. Immediately after adsorption the antigen present in the cells in two 250 ml. bottles when concentrated 20-fold

could only just be detected by complement fixation. Therefore, the cells from 10 bottles were pooled for each sample taken at intervals during a growth cycle and the antigen obtained from them was concentrated 100-fold in the following way. The medium from 10 bottles was decanted and pooled, and the monolayers washed twice and placed at −40°. At the end of the experiment, all the infected cells from each batch were suspended in 5 ml. diluent, disrupted, centrifuged and the deposit resuspended in 1 ml. diluent. The supernatant fluid will be referred to as 'soluble cellular antigen' and the deposit as 'sedimentable cellular antigen'. Pooled tissue culture medium from each sample was also centrifuged and the deposit, resuspended in 1 ml. of diluent, will be called 'extracellular sedimentable antigen'.

RESULTS

Antiserum prepared against yolk-sac virus was always used to titrate tissue culture antigens; antiserum against cell-cultured virus was used with yolk sac antigens. Thirty-six replicate titrations of the same antigen by complement fixation revealed a standard deviation of ±10% so that a two-fold difference of titre is significant at the 95% level. Trachoma and inclusion blennorrhoea viruses grown in yolk sac or cell culture were repeatedly titrated against homologous and heterologous antisera and, using unabsorbed antisera with either heated or unheated antigens, they could not be distinguished.

Table 1. *Effect of heat on different LB4 virus yolk-sac suspensions*

The antigens were titrated with LB1 antiserum.

Antigen	Titre		Decrease in titre
	Before heating	After heating	
V_1	3840	640	6-fold
V_2	960	480	2-fold
V_4	640	480	0
V_7	1280	960	0

Composition of group antigen

Effect of heat. Table 1 gives representative results obtained when four different LB4 yolk-sac suspensions were titrated with LB1 antiserum before and after heating. Only two suspensions underwent a significant decrease in titre after boiling. In twenty other titrations the titres of eight antigens were significantly decreased after heating, 11 were unchanged and only one was significantly enhanced. In six titrations with lymphogranuloma venereum (LGV) convalescent serum no alteration in titre occurred. Our results suggest that these viruses possess a heat-labile antigen, although this is not always demonstrable. In contrast to the findings of Barwell (1952) and others, boiling seldom enhanced the titre of the heat-stable antigen.

Effect of periodate. Table 2 summarizes the effect of different concentrations of potassium periodate on the titre of yolk-sac suspensions of two strains of inclusion blennorrhoea. The titres of both strains were decreased four-fold by all the concentrations tested; a constant fraction of antigen remained resistant to periodate. This

resistant fraction reacted equally with antisera against three strains of inclusion blennorrhoea, two strains of trachoma and LGV convalescent serum (Table 3).

Two different suspensions of a strain of trachoma virus grown in HeLa cells were treated with periodate and heated (Table 4). Heating alone did not decrease the antigen titres, measured with heterologous antiserum (LGV convalescent serum). Periodate treatment again decreased the antigen titres four-fold, leaving a periodate-resistant fraction which was heat-labile and still capable of reacting with heterologous antiserum.

Table 2. *Effect of different concentrations of KIO_4 on yolk-sac suspensions of two strains of inclusion blennorrhoea, titrated with LB1 antiserum*

Antigen	Concentration of KIO_4 (M)	Titre: Before treatment	Titre: After treatment
LB3	0·005	128	28
LB1	0·0125	96	24
	0·003	96	24
	0·0007	96	24

Table 3. *Reaction with heterologous antisera of KIO_4-resistant fractions of inclusion blennorrhoea and of trachoma viruses grown in HeLa cells*

Antigen	Titre: Before treatment	Titre: After treatment	Antiserum
LB1 suspension (*a*)	56	28	LB1
	56	20	LT1
	56	28	LB2
	56	16	LB4
	56	16	TE55
LB1 suspension (*b*)	64	24	LGV
TE55	56	12	LGV

Table 4. *Effect of heat and of KIO_4 on two different suspensions of trachoma virus (strain TE55) titrated with LGV convalescent serum*

Treatment	Titre of suspension (*a*)	Titre of suspension (*b*)
Control	56	64
Heated	56	48
0·025 M-KIO_4	12	24
0·025 M-KIO_4 then heated	0	6

Benedict & O'Brien (1956) demonstrated similar fractions in a soluble antigen extracted from feline pneumonitis virus; they showed the periodate-sensitive and fraction-resistants to be respectively a polysaccharide and a protein. By inference this is also true of the similar fractions of trachoma and inclusion blennorrhoea viruses.

Effect of ether. The antigen titre of an unheated suspension of LB 1 virus prepared from infected HeLa cells was not diminished by shaking with ether, and no antigen was detected in the ether extract. When, however, the suspension was boiled before ether treatment, a group reactive antigen was extracted. The ether-soluble antigen was insoluble in methanol and thus appeared to be a phosphatide. Although phospholipids and polysaccharides may fix complement *in vitro* some may

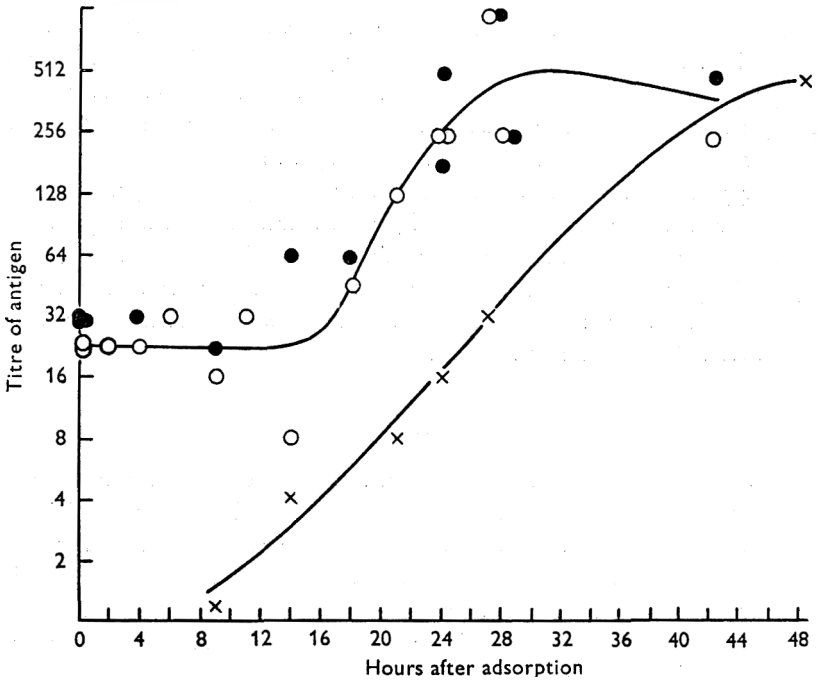


Fig. 1. Development of complement-fixing antigen during one growth cycle of the LB 1 strain of inclusion blennorrhoea in HeLa cells. Titres represent the total amount of antigen present in ten infected monolayers. ●—●, cellular soluble antigen; ○—○, cellular sedimentable antigen; × — ×, extracellular sedimentable antigen.

behave as haptens *in vivo*, only stimulating antibody formation when combined with protein. That a virus suspension must be boiled before a soluble antigen can be extracted with ether suggests that the antigen is strongly bound to a heat-sensitive component. The evidence suggests that the group antigen is a lipoprotein-polysaccharide complex.

Development of complement-fixing antigen of inclusion blennorrhoea virus in HeLa cells

On several occasions the complement-fixing antigen present in HeLa cell cultures was titrated at intervals during one growth cycle of the LB 1 strain of inclusion blennorrhoea virus. Figure 1 shows the results of a representative experiment in which unheated samples were titrated with homologous antiserum. Samples from normal HeLa cells concentrated in the same way did not fix complement with this serum. Each sample was also titrated after treatment with periodate. At each assay both soluble and sedimentable antigen proved heat-stable and a constant

proportion was destroyed by periodate. Thus, although the virus contains several antigens and the antiserum used was prepared against whole virus, our method could be used to examine only the fate of the lipoprotein polysaccharide group antigen.

During the first 14 hr. of infection there was little change in the total amount of cellular antigen. About half was deposited under the same conditions as whole virus particles, and the rest remained in the supernatant fluid. The amount of sedimentable antigen did not decrease at any time and its increase was not preceded by an increase in soluble antigen. Indeed, throughout the life cycle of the virus, a constant proportion of the group antigen was in the form of this smaller component, suggesting either that the virus is associated with a soluble product, or that a loosely bound outer antigen is stripped off during manipulation.

Antigen appeared to be released continuously throughout the growth cycle, until at 40 hr. after adsorption as much antigen was present in the supernatant fluid of infected cultures as could be released from the cells. In contrast, Furness & Fraser (1961) reported that at 42 hr. infectious extracellular virus only amounted to 1 % of the infectious virus within the cells. It appears that released virus is rapidly inactivated at 37° but remains antigenic.

DISCUSSION

Our experiments suggest that trachoma and inclusion blennorrhoea viruses contain antigens similar to the lipoprotein polysaccharide complex of feline pneumonitis described by Benedict & O'Brien (1956). The complement-fixing component of an intact particle is likely to be at the surface; and Jenkin (1960), working with meningopneumonitis, believed that in the intact particle the group antigen resided in the cell wall. Therefore, study of the group antigen may reveal the behaviour of the cell wall of the virus during its life cycle.

The life cycles of members of the psittacosis-lymphogranuloma venereum (LGV) group of viruses have been studied by many workers from the point of view of morphology and development of infective particles (see Wenner, 1958, for recent review; Higashi, Natake & Fukada, 1959; Furness & Fraser, 1962); less work has been done on the development of complement-fixing antigens. Bedson & Gostling (1954) studied the production of group antigen in the spleens of mice infected with psittacosis; Officer & Brown (1960) measured the group antigen formed during the growth of psittacosis virus in cell culture. In neither study was antigen detected before the 16th hr. after infection.

Our experiments establish two facts. First, the antigen can be detected throughout the growth cycle and, secondly, the amount of antigen increases at least 6 hr. before all the adsorbed virus increases in infectivity (Furness & Fraser, 1962). Investigation of other members of the group by several different techniques indicates that although the viruses are not infectious during the first 14 hr. after adsorption they do not lose their identity. Thus, elementary bodies of meningopneumonitis virus staining with Macchiavello's stain were seen in L cells up to 14 hr. after infection (Higashi *et al.* 1959); particles containing DNA were detected up to 4 hr. after infection of human amnion cells with psittacosis virus (Pollard, Starr, Moore & Tanami, 1960); and psittacosis virus particles combining

with fluorescent antibody were present in the cytoplasm of mouse-embryo liver cells 1, 3, 5 and 8 hr. after infection (Buckley, Whitney & Rapp, 1955). Neither Bedson & Gostling (1954) nor Officer & Brown (1960) detected complement-fixing antigens of psittacosis virus until 16 hr. after the start of the growth cycle, but in neither study was the amount of virus adsorbed detected by complement fixation, suggesting that the methods used were insufficiently sensitive to detect antigen until some multiplication had occurred. By concentrating the virus we were able to detect the antigen adsorbed at the start of the growth cycle, and to show that antigen persisted in the cells during the early stages.

The amount of complement-fixing antigen increased when the large basophil particles, rich in RNA (Pollard *et al.* 1960; Furness, Henderson, Csonka & Fraser, to be published) increased in amount. These large forms are the only structures with which the sedimentable cellular antigen could be associated; Buckley and co-workers (1955) demonstrated that the large forms of psittacosis virus react with fluorescent antibody. Twenty-four hours after infection, when the amount of antigen has reached its maximum, less than 10 % of the adsorbed virus is infectious (Furness & Fraser, 1961). Much of the antigen later associated with elementary bodies is apparently formed earlier during the development of the large forms.

Our results support Higashi's interpretation of the life cycle of this group of micro-organisms (Higashi, 1959), since the antigen persists unchanged during the non-infective phase. The large forms, which are rich in RNA, may be regarded as a vegetative stage during which most synthesis, including that of the cell-wall components, takes place.

We are grateful to Miss Elizabeth Fraser for supplying HeLa cell cultures.

Sera from patients with lymphogranuloma venereum were generously supplied by Dr Sylvia Read, London Hospital, Whitechapel, and Dr G. Csonka, St Mary's Hospital, London.

REFERENCES

Barwell, C. F. (1952). Some observations on the antigenic structure of psittacosis and lymphogranuloma viruses. II. Treatment of virus suspensions by various reagents and the specific activity of acid extracts. *Brit. J. exp. Path.* **33**, 268.

Bedson, S. P. (1936). Observations bearing on the antigenic composition of psittacosis virus. *Brit. J. exp. Path.* **17**, 109.

Bedson, S. P., Barwell, C. F., King, E. J. & Bishop, L. W. J. (1949). The laboratory diagnosis of lymphogranuloma. *J. clin. Path.* **2**, 241.

Bedson, S. P. & Gostling, J. V. T. (1954). A study of the mode of multiplication of psittacosis virus. *Brit. J. exp. Path.* **35**, 299.

Benedict, A. A. & O'Brien, E. (1956). Antigenic studies on the psittacosis-lymphogranuloma venereum group of viruses. II. Characteristics of complement-fixing antigen extracted with sodium lauryl sulfate. *J. Immunol.* **76**, 293.

Buckley, S. M., Whitney, E. & Rapp, F. (1955). Identification by fluorescent antibody of developmental forms of psittacosis virus in tissue culture. *Proc. Soc. exp. Biol., N.Y.* **90**, 226.

Fulton, F. & Dumbell, K. R. (1949). The serological comparison of strains of influenza virus. *J. gen. Microbiol.* **3**, 97.

Furness, G. & Fraser, E. F. (1962). One-step growth curves for inclusion blennorrhoea virus in Hela cell monolayers. *J. gen. Microbiol*, **27**, 299.

Furness, G., Graham, D. M. & Reeve, P. (1960). The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. *J. gen. Microbiol.* **23**, 613.

HIGASHI, N. (1959). Some fundamental differences of the mechanism of multiplication between psittacosis group viruses and pox viruses. *Ann. Report Inst. Virus Research, Kyoto Univ.*, Series B, **2**, 1.

HIGASHI, N., NOTAKE, K. & FUKADA, T. (1959). Growth characteristics of the meningopneumonitis virus in strain L cells. *Ann. Report Inst. Virus Research, Kyoto Univ.*, Series B, **2**, 23.

HILLEMAN, M. R. & NIGG, C. (1946). Studies on lymphogranuloma venereum complement-fixing antigens. III. The solubility in ether of an active fraction. *J. Immunol.* **53**, 201.

JENKINS, H. M. (1960). Preparation and properties of cell walls of the agent of meningopneumonitis. *J. Bact.* **80**, 639.

JONES, B. R. (1961). TRIC virus infection in London. *Trans. Ophthal. Soc. U.K.* **8**, 367.

JONES, B. R., COLLIER, L. H. & SMITH, C. H. (1959). Isolation of virus from inclusion blennorrhoea. *Lancet*, i, 902.

MONSUR, K. A. & BARWELL, C. F. (1951). Observations on the antigenic relationship between the virus of enzootic abortion of ewes and viruses of the psittacosis-lymphogranuloma group. *Brit. J. exp. Path.* **32**, 414.

OFFICER, J. E. & BROWN, A. (1960). Growth of psittacosis virus in tissue culture. *J. infect. Dis.* **107**, 283.

POLLARD, M., STARR, T. J., MOORE, R. W. & TANAMI, Y. (1960). Cytochemical changes in human amnion cells infected with psittacosis virus. *Nature, Lond.* **188**, 770.

REEVE, P. & GRAHAM, D. M. (1962). *J. gen. Microbiol.* **27**, 177.

ROSS, M. R. & GOGOLAK, F. M. (1957). The antigenic structure of psittacosis and feline pneumonitis viruses. I. Isolation of complement-fixing antigens with group and species specificity. *Virology*, **3**, 343.

SIGEL, M. M. & POLLIKOFF, R. (1953). Reduction of group reactivity of complement-fixing antigen of meningo-pneumonitis virus by potassium periodate. *Proc. Soc. exp. Biol., N.Y.* **84**, 517.

T'ANG, F. F., CHANG, H. L., HUANG, Y. T. & WANG, K. C. (1957). Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. *Chin. med. J.* **75**, 429.

VOLKERT, M. & CHRISTENSEN, P. M. (1954). Studies on ornithosis in Denmark. *Acta path. microbiol. scand.* **35**, 584.

WENNER, H. A. (1958). Psittacosis-lymphogranuloma venereum group of viruses. *Advanc. Virus Res.* **5**, 39.

J. gen. Microbiol. (1962), 27, 509–520
With 2 plates
Printed in Great Britain

The Establishment of Vesicular-Arbuscular Mycorrhiza under Aseptic Conditions

By BARBARA MOSSE

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 26 *July* 1961)

SUMMARY

The establishment of vesicular-arbuscular mycorrhizal infections by inoculation with germinated resting spores of an *Endogone* sp. was investigated under microbiologically controlled conditions; pure two-membered cultures were obtained for the first time.

Seedlings were grown in a nitrogen-deficient inorganic salt medium; in these conditions the fungus failed to form an appressorium and to penetrate the plant roots unless a *Pseudomonas* sp. was also added. Adding soluble nitrogen to the medium completely inhibited root penetration, even in the presence of the bacteria. Various sterile filtrates could be used to replace the bacterial inoculum but these substitutes induced only few infections per plant.

Mycorrhizal roots grew more vigorously than non-mycorrhizal roots of the same seedling. They were longer and more profusely branched. At first mycorrhizal infections were predominantly arbuscular, but many prominent vesicles developed as the seedlings declined, and then the fungus grew out of infected roots and colonized the agar. The fungus could not be subcultured without a living host.

The possible interpretation of these results is considered with reference to the specialized nutritional conditions under which test plants were grown.

INTRODUCTION

For a critical study of the effects of vesicular-arbuscular mycorrhiza on plant growth, typical infections must be produced under controlled microbiological conditions. Some progress has been made towards this with the isolation of four different fungi able to cause such infections. Two of these, an unidentified species at present called *Rhizophagus* sp. (Barrett, 1947, 1958), and a strain of *Pythium ultimum* (Hawker, Harrison, Nicholls & Ham, 1957) are available in pure culture, whereas the others, both *Endogone* spp. (Mosse, 1953, 1956, 1959*a*; Gerdemann, 1955) were obtained as soil-borne spores. In successful inoculation experiments, test plants were grown in sterilized soil or in a liquid medium in open containers. Repeated attempts to produce normal vesicular-arbuscular mycorrhiza in an angiosperm species under aseptic conditions have so far failed (Hawker *et al.* 1957; Barrett, 1959; Mosse, 1959*b*). Hepden (1960) appears to have had some success with fern sporophytes grown in sand culture but the absence of other

microbial contaminants in the successfully inoculated plants was not established. All the evidence suggests that very specialized conditions, or perhaps even the presence of some other micro-organism, are needed to establish vesicular-arbuscular infections in culture. The inoculation experiments described below confirm this suggestion. The excised, surface-sterilized, resting spores of a sporocarp-forming *Endogone* sp. were used as inoculum. Under aseptic conditions the germ tubes of this fungus did not penetrate the roots of test plants, but the presence of certain soil bacteria enabled them to do so when the plants were deficient in nitrogen. Some, but much less root penetration was also induced by 'Pectinol', a commercial pectinase enzyme preparation (Rohm and Haas Co., Philadelphia, Pennsylvania, U.S.A.), by ethylenediaminetetra-acetic acid (EDTA), a chelating agent, and by cell-free filtrates of culture solutions in which plants and bacteria had been grown together. This makes it possible to synthesize vesicular-arbuscular mycorrhiza in pure two-membered cultures.

METHODS

In experiments of this kind three factors require consideration: (1) the provision of sterile test plants; (2) the composition of the medium in which both plant and fungus must grow; (3) the preparation of a sterile inoculum.

Test plants. Seedlings of *Trifolium parviflorum* were used for the systematic inoculation experiments, and *T. glomeratum*, *T. pratense*, *T. subterraneum*, *Dactylis glomerata*, wheat (*Triticum vulgare*), cucumber (*Cucumis sativus*) and onion (*Allium cepa*) seedlings were also used to test the general applicability of the results.

Clover seeds were sterilized in concentrated sulphuric acid, chilled, and germinated on agar plates, by a technique described by Nutman (1949). All other seeds were sterilized for 15 min. in 10 ppm ethyl mercury phosphate. They were then rinsed in fifteen changes of sterile water, and were incubated at 15° on 0·75 % agar in water (w/v) again. After germination, seedlings were transferred to agar slopes in sterile test tubes, which were kept in a greenhouse.

Culture medium. All test plants were grown in a nitrogen-deficient inorganic salt medium formulated by Jensen (1942) and used extensively in nodulation studies. It contained g./l. of distilled water: 1, $CaHPO_4$; 0·2, K_2HPO_4; 0·2, NaCl; 0·2, $MgSO_4.7H_2O$; 0·1, $FeCl_3$; 7·5, shredded agar. The medium was adjusted to pH 6·3 before autoclaving at 120° for 20 min.

Inoculum. An uncontaminated inoculum of germinated resting spores of an *Endogone* sp. was obtained as previously described (Mosse, 1959*a*). Spores excised from the sporocarps were sterilized in two changes of a freshly prepared 2 % (w/v) solution of Chloramine T containing 200 ppm streptomycin. After washing, 20–30 sterilized spores were transferred to a small cellophan disk, which was placed on a large piece of sterile cellophan spread over a soil agar plate containing 0·2 g. soil dispersed in 5 ml. 0·75 % (w/v) agar in water and containing 0·001 % crystal violet. After incubation for 5 to 6 days at 20° the small cellophan disks, now covered with a network of anastomosing germ tubes, were tested for freedom from bacterial contaminants by incubating them on nutrient agar for 24 hr. They were also examined microscopically and any with fungal contaminants were discarded. More than 90 % of the disks carried a pure inoculum, which was introduced, without disturbing the mycelial network, by placing the small cellophan disks, with the spore-bearing surface uppermost, near to an actively growing seedling root.

RESULTS

Conditions necessary for the establishment of infections

The preparation of the inoculum is laborious, and as the results looked for were qualitative rather than quantitative, a limited replication was considered to be sufficient. Each inoculation test was repeated at least three times, often on two or more separate occasions. In all over 800 inoculations were made, yielding reasonably consistent results. The most variable factor was a seasonal difference in the growth rate of test seedlings which affected the time required for the establishment of infections. Infection was favoured by temperature and light conditions which promoted vigorous seedling growth. A 12-hr. day length was maintained by supplementary fluorescent lighting during the winter, and temperatures of 20–25° during part of this period assisted infection. Except for four strains of subterranean clover, all the plant species tested became mycorrhizal (Table 1), under certain specialized conditions which are discussed below.

The bacterial factor. The inoculation of sterile seedlings with sterile germinated spores never produced a mycorrhizal infection. Altogether about 50 two-membered cultures of this kind were examined. The fungus did not penetrate the roots even when they were damaged mechanically. In the earliest experiments, however, in which the sterility of the inoculum had not been checked, a few test plants became mycorrhizal, and all these cultures also contained a bacterial contaminant, often a *Pseudomonas* sp. Two pseudomonad isolates (No. 1 and 2) were obtained in pure culture. Both grew well on beef-extract peptone yeast agar, on which No. 2 produced a strongly fluorescent pigment. Both pseudomonads induced the changes necessary for fungal penetration of the roots (Table 1), but neither directly affected the germination of Endogone spores or the growth of the germ tubes. Quite a small bacterial inoculum sufficed to mediate infections, and it was immaterial whether the pseudomonads were added together with the fungal inoculum or later. Mycorrhizal infections were also produced in liquid culture with the aid of a pseudomonad inoculum. No bacteria were found within mycorrhizal roots.

Table 1 shows that other bacteria also induced the changes leading to infection. Two strains of *Pseudomonas marginalis*, both with pectolytic activity, had this effect, but a nodule-forming strain of a *Rhizobium* sp. had not. This aspect of the problem was not further pursued, but experiments designed to replace the bacterial factor by cell-free extracts give some indication of ways in which the bacterial inoculum may act. Table 2 gives a summary of some of these experiments.

Of the various cell-free extracts tried, only porcelain-filtered suspensions of the bacteria in distilled water (Expt. 1*b*, Table 2), and filtrates from dual cultures of plant and bacteria grown in a nitrogen-deficient liquid medium (Expt. 1*c* and *d*, Table 2), led to the establishment of infections. It seemed that the active factor might be some enzyme produced by bacteria when lacking nitrogen; two crystalline proteolytic enzymes, pepsin and trypsin, were however without effect (Expt. 2, Table 2). The role of pectolytic enzymes in the penetration processes of many pathogenic micro-organisms (Wood, 1955), and of nodule bacteria (Fåhraeus & Ljunggren, 1959; Ljunggren, 1961) suggested tests with Pectinol, a commercial enzyme preparation that is both pectolytic and cellulolytic (Jackson, 1959). The Pectinol, containing the active enzymes adsorbed on an inert carrier, was sus-

pended in water, filtered to remove the insoluble carrier and then sterilized by filtration through a porcelain candle. At concentrations from 0·02 down to 0·0002 % (w/v) it was the most effective of the sterile filtrates. Concentrations of Pectinol higher than 0·02 % were not more effective, and caused discoloration and stunting of the clover roots. On the assumption that the Pectinol acted as a mild macerating agent, tests were made with ethylenediaminetetra-acetic acid (EDTA) which has

Table 1. *The effect of bacterial inoculation and soluble nitrogen on the establishment of Endogone infections in sterile seedlings*

Test plant	Bacterial inoculum	Additional nitrogen source	No. of plants with vesicular-arbuscular infections
Trifolium parviflorum	None	None	0/56
	Pseudomonas sp.	,,	30/30
T. parviflorum (in liquid culture)	*Pseudomonas* sp.	,,	6/6
T. parviflorum	*Pseudomonas* sp., isolate 2	,,	15/15
	P. marginalis, No. 506*	,,	5/5
	P. marginalis, No. 667*	,,	3/3
	Chance contamination	,,	60/81
	Rhizobium sp.	,,	0/3
	Pseudomonas sp.	0·05 % KNO_3	0/8
		0·05 % $(NH_4)_2SO_4$	0/8
		0·05 % urea	1/6†
		0·05 % asparagine	1/8†
T. pratense	None	None	0/5
	Pseudomonas sp.	,,	5/5
T. glomeratum	None	,,	0/3
	Pseudomonas sp.	,,	3/3
Dactylis glomerata	None	,,	0/5
	Pseudomonas sp.	,,	6/10
Wheat	None	,,	0/3
	Pseudomonas sp.	,,	3/10
Onion	None	,,	0/3
	Pseudomonas sp.	,,	5/5
Cucumber	None	,,	0/5
	Pseudomonas sp.	,,	10/18
T. subterraneum 4 strains	None	,,	0/5
	Pseudomonas sp.	,,	0/5

Pseudomonas sp. isolate 1 was used as bacterial inoculum unless otherwise stated.
* Supplied by Dr R. A. Lelliott, Plant Pathology Laboratory, Harpenden, Hertfordshire.
† Infections developed after 10 weeks.

also been used in maceration (Humphries & Wheeler, 1960; Ginzburg, 1961). At low concentrations EDTA, although acting more slowly than Pectinol also assisted in the infection process of Endogone spores, but at concentrations higher than 0·0002% it did not, and damaged the roots (Expt. 4, Table 2). Tomlinson & Webb (1960)

reported a similar action of EDTA which enabled *Erisyphe polygoni* to infect turnip tissue treated with zinc sulphate. Various mixtures of Pectinol, EDTA, trypsin and pepsin were tried, but none proved more effective than Pectinol alone. Autoclaved Pectinol and autoclaved filtrates from dual cultures of plant and bacteria to which 0·1 % (w/v) pectin had been added were also effective sometimes (Expt. 1*d*, 3, Table 2) but, as with EDTA, infections developed only slowly.

Table 2. *The effect of sterile filtrates on the establishment of* Endogone *infections in* Trifolium parviflorum

Substances tested	Method of sterilization	Final concentration (%)	No. of plants with vesicular-arbuscular infections
(1) Culture filtrates of *Pseudomonas* sp. (isolate 1)			
(*a*) Grown for 2 days in beef-extract yeast peptone broth	A	10	0/5
	S	10	0/3
	C	10	0/4
(*b*) Grown in nutrient broth; centrifuged and suspended in distilled water	A	10	0/6
	S	10	0/3
	C	10	2/10
	D	10	0/15
(*c*) Grown with clover seedling for 2 weeks in Jensen's medium	A	10	0/3
	C	10	5/14
	C	50	2/4
(*d*) Grown with clover seedling for 2 weeks in Jensen's medium +0·1 % (w/v) pectin	A	10	4/11*
	C	10	8/15
(2) Proteolytic enzymes			
(*a*) trypsin	C	10^{-3}	0/3
		10^{-1}	0/2
(*b*) pepsin	C	10^{-3}	0/5
		10^{-1}	0/2
(3) Pectinol 100 D	C	0·1	1/3 some root
		2×10^{-2}	12/16 damage
		2×10^{-3}	3/5
		2×10^{-4}	4/6
	A	2×10^{-2}	2/5*
(4) Ethylenediaminetetraacetic acid (EDTA) neutralized with NaOH	A	2×10^{-2}	0/4 some root
		2×10^{-2}	0/5 damage
		2×10^{-4}	3/5* roots slightly
		2×10^{-5}	3/5* stunted

Methods of sterilization A = autoclaved at 120° for 20 min.; S = Seitz filtered; C = filtered through porcelain candle; D = sonically disintegrated and filtered through porcelain candle.
* Infections developed slowly.

Although indistinguishable in anatomical details, infections established with the aid of a bacterial inoculum were always more widespread than those induced with sterile filtrates (Table 3). Adding larger amounts of filtrate than in Table 3 did not increase the number of infections; it is therefore possible that the active factor becomes adsorbed or otherwise inactivated during the experiment, or that the optimum concentration is low. It was thought that the number of infections might be related to plant size and photosynthetic area, which was increased as a result of adding the bacterial inoculum. Applying urea to the leaves, which increased plant

size, did not however increase the number of infections, although it stimulated fungal growth in the agar once an infection had taken place, and there were more points of contact between the mycelium and the root system.

The effect of soluble nitrogen in the medium. In addition to the bacterial factor, the supply of nitrogen in the medium is important in the establishment of Endogone infections. The mineral salt medium used contains a very small amount of nitrate as impurity; 1·5 μg. in 10 ml. solution. A more important nitrogen source was the shredded agar which contributed 150 μg. total-N in 10 ml. of the medium; less than one quarter of this was directly available to the plant. The rest of this nitrogen in the agar was probably in the form of protein-like impurities (*Spec. Rep. Soc. gen. Microbiol.* 1956) some of which can be made available to the plant by microbial and enzyme action. In such a nitrogen-deficient medium the small-seeded clover species began to show symptoms of nitrogen deficiency after 2–3 weeks. To overcome this, potassium nitrate, ammonium sulphate, asparagine and urea were tried as additional nitrogen sources at 0·05 % (w/v). Such additions improved plant growth, but completely inhibited the establishment of Endogone infections, even in the presence of bacteria (Table 1). A few infections developed after 10 weeks or more, when the plants were again showing symptoms of nitrogen starvation.

Table 3. *The effect of bacterial inoculations and sterile filtrates on the intensity of mycorrhizal infection in sterile seedlings of* Trifolium parviflorum *inoculated with a species of* Endogone

Aid to infection	No. of plants examined	Mean no. of mycorrhizal roots per plant	Mean no. of entry points per plant	Mean no. of entry points per infected root
Pseudomonas sp., isolate 1	10	5·2	22·0	4·2
P. marginalis, No. 506	5	5·0	20·0	4·0
Chance contamination	20	4·1	17·0	4·1
Sterile filtrate from dual culture of plant and *Pseudomonas* sp., isolate 1	10	2·3	2·8	1·2
Pectinol	12	1·8	3·7	2·1
EDTA	3	2·0	3·0	1·5

The progress of infection

Infection depends on chance contact between a root and a growing hypha, and there was no evidence that hyphae are attracted towards the root surface. Pregermination of the Endogone spores was essential but inoculum size had no obvious effect. Inoculation with a single germinated spore can lead to infection. Without the modifying influence of bacteria, or one of the substances listed, appressoria did not form. Hyphae then grew around and along the root surface without forming any firm attachment, until spore reserves were exhausted. Nevertheless, the seedling exerts some stimulatory effect, because without it growth of the germ tubes is limited, septa arise, and the protoplasm is retracted into the spore. Such spores, when suitably stimulated, can then germinate a second and third time. Under favourable conditions the first infections occur 5–10 days after inoculation, but the process may take 3 weeks or even longer. Penetration stimulates the growth of the infected root and of distal parts of the mycelial network. Hyphae grow beyond the cellophan disk and into the agar, and when they encounter another root establish

further infections. Young infections are mainly in the form of arbuscules and of intercellular distributive hyphae, and are usually of limited extent. Root growth commonly outstrips the spread of the infection and, as in natural infections, the actively growing root tip is never invaded. Infections were rarely found in the main root, and there was no internal spread from one root to another. Usually less than a third of the total root system became infected. Anatomically, aseptically produced infections (Pl. 1, fig. 1–5) are in every way comparable to those found in field plants. The first vesicles often occur close to a penetration point, indicating some association with more advanced stages of infection. As the infection progresses vesicles become more abundant, starch reserves disappear, and arbuscules break down till eventually no sign of them remains (Pl. 1, fig. 5). After 3–6 weeks test plants begin to decline and about this time the character of the Endogone infections undergoes a dramatic change. Coarse, macroscopically visible hyphae grow out from many parts of the infected root system (Pl. 2, fig. 6) and rapidly colonize the agar. Often such hyphae arise from prominent vesicles in the slowly disintegrating primary cortex. This observation supports the view of Schrader (1958) on the possible importance of vesicles as infective propagules. Anatomical evidence and the appearance of the plants suggest that, at this point, the relationship between plant and fungus changes to one of parasitism. In its growth the external mycelium resembles cultures of *Rhizophagus* sp. kindly supplied by Dr J. T. Barrett. The characteristic asexual spores produced by the external mycelium may eventually reach 75 μ in diameter. In spite of its vigorous growth the mycelium could not be subcultured on hemp seed agar (on which the *Rhizophagus* grows) or on any other standard fungal medium.

The maintenance of two-membered cultures

Once a pure two-membered culture is established it can be propagated in several ways. Fresh seedlings can be planted on an infected agar slope that has been colonized by the Endogone mycelium, and new infections will develop in 5 to 10 days. The original mycorrhizal seedling need not be present. Alternatively infected seedlings can be transplanted to fresh agar slopes which will in turn become colonized by hyphae arising from the infected root system, or pieces of infected roots can be used as inoculum. As, however, infected roots are distributed at random and are not easily recognizable without staining, the best way of selecting infective material is to plate out the entire root system in Jensen's medium and incubate the plates at 25° for 36 hr. New hyphae arise from those parts of the root system that can be used successfully as inoculum. Where the infection is predominantly in the arbuscular stage no new hyphae arise; all such roots tested were non-infective. This agrees with the observations of Hawker *et al.* (1957), and supports the suggestion that the vesicular and arbuscular stages of an endotrophic infection may represent different phases in the balance between host and endophyte.

The growth of infected seedlings

Mycorrhizal infection did not obviously improve the macroscopic appearance of test seedlings. Individual mycorrhizal roots, however, were often strikingly more vigorous than uninfected roots of the same seedling (Pl. 2, fig. 7*a*, *b*). The length

and number of branches of all first-order laterals were recorded in twenty seedlings selected at random from various successful inoculation experiments; in some, mycorrhizal infections had been established with the aid of a bacterial inoculum, in others with sterile filtrates. All the seedlings were showing signs of nitrogen starvation when they were harvested. Figure 1 shows the mean length of mycorrhizal and non-mycorrhizal roots. Only the first twenty-one laterals are included because in the more distal roots few mycorrhizal infections developed, probably because the inoculum was placed near the proximal end of the root system as indicated. Points near this position represent the oldest infections; those near the right-hand side of the diagram represent more recently infected roots. Second-order laterals were added to the length of the first-order lateral from which they arose. Seventy-nine % of mycorrhizal roots were longer than the mean of the nearest uninfected roots above and below them. Often they were two, three or even four times as

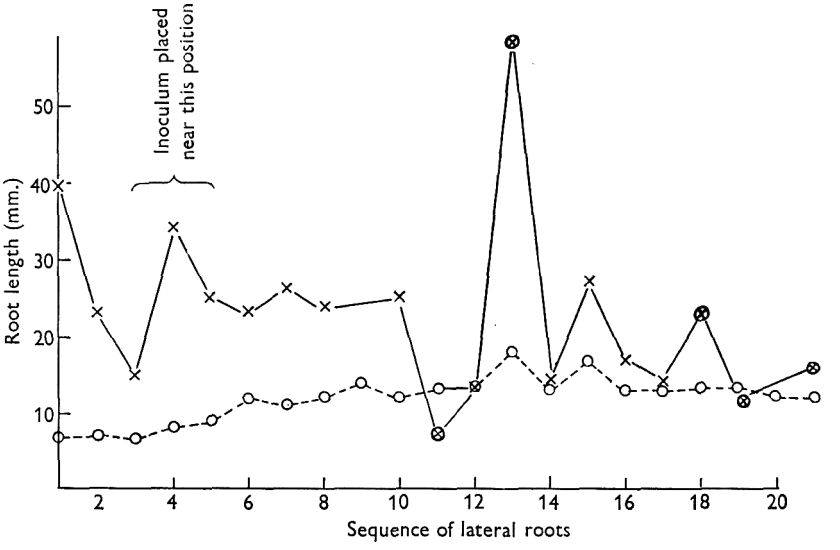


Fig. 1. Mean length of mycorrhizal and non-mycorrhizal first-order lateral roots in twenty seedlings of *Trifolium parviflorum*. O----O, non-mycorrhizal roots; × — ×, mycorrhizal roots; ⊗, point based on a single measurement.

long, and thus clearly outside the normal size range of uninfected roots. Mycorrhizal roots were also more profusely branched. There were thirty-three second-order laterals on a total of fifty-nine mycorrhizal roots, but only nine second-order laterals on 361 non-mycorrhizal ones.

The Pseudomonas inoculum used to establish mycorrhizal infections greatly improved plant growth and increased dry weight by up to 100%; Pl. 2, fig. 8, shows typical seedlings. The mechanism of this effect was further investigated by chemical analysis and will be described elsewhere. For a better understanding of the conditions leading to mycorrhizal infections the following information is relevant. The bacterial inoculum benefits plant growth because the bacteria degrade nitrogen-containing impurities in the agar and thus make extra nitrogen available to the plant. Both pepsin and trypsin, and to a lesser extent Pectinol, had a similar effect on plant growth. The bacterial effect is therefore probably connected with the

development of proteolytic enzymes in the bacteria. A range of soil saprophytic fungi also made extra nitrogen available to the seedling, as did the two mycorrhizal fungi *Rhizophagus* sp. and a strain of *Pythium ultimum* kindly supplied by Dr L. Hawker; but under the experimental conditions provided none of these fungi penetrated the clover roots. By contrast the extensive external mycelium developing from roots infected with Endogone did not exert a similar proteolytic effect on agar nitrogen, for it did not noticeably improve plant growth. A non-nodulating strain of *Rhizobium* sp. also had no effect on the availability of agar nitrogen.

DISCUSSION

The purpose of the inoculation experiments was to establish vesicular-arbuscular mycorrhiza under controlled microbiological conditions. Beneficial effects of such infections on plant growth have been reported by Mosse (1957), Peuss (1958), Bayliss (1959), Meloh (1961) and others; Meloh also recorded an increased uptake of dihydrostreptomycin. These results are open to the criticism that impure inocula were used to establish the infections, and that some or all of the observed effects were caused by other micro-organisms. This criticism does not apply to the present experiments in which a stimulatory effect of mycorrhizal infection on root growth was demonstrated under controlled microbiological conditions. Both root extension and branching were favourably affected. The possibility that mycorrhizal infection occurs in roots predisposed to, or already engaged in, vigorous growth is remote. Growth of the Endogone germ tubes is very limited and therefore the site of at least the first infections is determined, not by fungal selection, but almost entirely by the positioning of the inoculum. Although these observations establish that root growth can be stimulated by mycorrhizal infection, there is little indication how this occurs. The culture medium used in the inoculation experiments is very unbalanced, containing abundant phosphate and very little nitrogen, so that the latter almost certainly acts as a limiting factor in the later stages of seedling growth. Any improvements in growth therefore immediately suggest either an extra supply of nitrogen or a more efficient use of available sources. There were two possible sources of extra nitrogen in these experiments; i.e. nitrogenous impurities in the agar which were resistant to the proteolytic action of the *Pseudomonas* sp., or gaseous nitrogen in the atmosphere. Clearly if either of these sources were used, the amounts involved were small, because the stimulatory effects did not extend beyond the immediately affected roots. For this reason it would be desirable to improve culture techniques to obtain more abundantly infected root systems.

The main obstacle to more extensive infection is the inability of the *Endogone* sp. to infect aseptic seedlings, and lack of knowledge concerning the action of the bacterial inoculum and the sterile filtrates. Any satisfactory explanation must account for the following observations. (1) Infections occurred only under conditions of nitrogen starvation. The responsive units were probably the bacteria which developed proteolytic activity. No infections occurred when only plant and fungus were grown together under the same conditions. The mediating action of the bacteria is, however, unlikely to rest solely on the provision of proteolytic enzymes, because it could not be replaced by either pepsin or typsin. Neither does it rest on some material in the agar, i.e. some trace element or amino acid, because infections

were also produced in liquid culture without agar. The bacterial effect might depend on the destruction of some inhibitory substance present in the plant or the medium, but the destruction of such a hypothetical substance would have to be inhibited by soluble nitrogen. (2) Candle-filtered solutions of Pectinol, and of the liquid medium in which clover seedlings had been grown together with a *Pseudomonas* sp. had some ability to mediate Endogone infections, but induced fewer infections than the bacteria. (3) EDTA, autoclaved Pectinol and autoclaved culture filtrates sometimes mediated infections, and their activity could not depend on enzyme action.

The most likely explanation seems to be that the mediating substances act on the structure of the cell wall, modifying or dissolving its pectic components. This may be done by direct enzyme action involving pectolytic, proteolytic and possibly also cellullolytic enzymes, by removal through chelation of the calcium cross-linkages affecting both pectic and protein cell wall components (Wood, 1960; Ginzburg, 1961), or by altering the proportion of water-soluble to water-insoluble pectin, thus affecting cell wall plasticity. Increases in cell-wall plasticity have been reported as a result of treatment with autoclaved Pectinol (Jackson, 1959) and with growth substances (Albersheim & Bonner, 1959; Jansen, Jang, Albersheim & Bonner, 1960). Many of these withstand autoclaving. The proportion of water-soluble to water-insoluble pectin can also be affected by the availability of calcium and, as was shown by Edgington, Corden & Dimond (1961), this can affect the susceptibility of plant tissue to fungal attack. Although resistance of the clover seedlings to infection may have been increased by the high calcium content of Jensens' medium, it is unlikely to have been entirely caused by it, because in earlier experiments (Mosse 1959*a*) other seedlings grown in tap water agar also resisted infection, indicating that the *Endogone* sp. genuinely lacks penetrating ability.

Vesicular-arbuscular endophytes have long been regarded as a group of highly specialized fungi. To the evidence of their great geological age, their high degree of adaptation to a restricted habitat and their near obligate relationship with a living host (which remains a characteristic attribute of most endophytes in spite of the artificial culture of two species) there can now be added, for the *Endogone* sp. used in the present experiments, a dependence on other soil micro-organisms for the processes of spore germination (Mosse, 1959*a*) and root penetration. The usual pattern is a dependence on root exudates for these two processes which are important stages in the establishment of fungal infections (Flentje, 1959).

The external mycelium which developed abundantly from Endogone-infected roots showed a remarkable similarity to cultures of *Rhizophagus* sp. The two fungi differ in their ability to grow on defined media, to break down nitrogenous impurities in the shredded agar, and to develop mycorrhizal infections under the conditions of the present experiments. In addition, infection with Endogone stimulated root growth under conditions where nitrogen was limiting growth. One is led to speculate whether the endophytic habit may be related to some special nitrogen requirement of the fungus. With a technique for producing mycorrhizal infections under controlled experimental conditions, this and other nutritional aspects of mycorrhizal infections can now be investigated.

I thank Dr P. S. Nutman and members of the Microbiology Department for their encouragement and for many helpful suggestions made in the course of this work.

REFERENCES

ALBERSHEIM, P. & BONNER, J. (1959). Metabolism and hormonal control of pectic substances. *J. biol. Chem.* **234**, 3015.

BARRETT, J. T. (1947). Observations on the root endophyte Rhizophagus in culture. *Phytopathology*, **37**, 359.

BARRETT, J. T. (1958). Synthesis of mycorrhiza with pure cultures of Rhizophagus. *Phytopathology*, **48**, 391.

BARRETT, J. T. (1959). Isolation, culture and host relation of the phycomycetoid vesicular-arbuscular mycorrhizal endophyte Rhizophagus. *Proc. 9th int. bot. Congr.* **2**, 1725.

BAYLISS, G. T. S. (1959). Effect of vesicular-arbuscular mycorrhizas on growth of *Griselinia littoralis* (Cornaceae). *New Phytol.* **58**, 274.

EDGINGTON, L. V., CORDEN, M. E. & DIMOND, A. E. (1961). The role of pectic substances in chemically induced resistance to Fusarium wilt of tomato. *Phytopathology*, **51**, 179.

FÅHRAEUS, G. & LJUNGGREN, H. (1959). The possible significance of pectic enzymes in root hair infection by nodule bacteria. *Physiol. Plant.* **12**, 145.

FLENTJE, N. T. (1959). The physiology of penetration and infection. In *Plant pathology problems and progress* 1908–58, ed. C. S. Holton and others. Madison, U.S.A.: University of Wisconsin Press.

GERDEMANN, J. W. (1955). Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. *Mycologia*, **47**, 619.

GINZBURG, B. Z. (1961). Evidence for a protein gel structure cross-linked by metal cations in the intercellular cement of plant tissue. *J. exp. Bot.* **12**, 85.

HAWKER, L. E., HARRISON, R. W., NICHOLLS, V. O. & HAM, A. M. (1957). Studies on vesicular-arbuscular endophytes. I. A strain of *Pythium ultimum* Trow. in roots of *Allium ursinum* L. and other plants. *Trans. Brit. mycol. Soc.* **40**, 375.

HEPDEN, P. M. (1960). Studies in vesicular-arbuscular endophytes. II. Endophytes in the Pteridophyta, with special reference to Leptosporangiate ferns. *Trans. Brit. mycol. Soc.* **43**, 559.

HUMPHRIES, E. C. & WHEELER, A. W. (1960). The effects of kinetin, gibberellic acid and light on the expansion and cell division in leaf discs of dwarf bean (*Phaseolus vulgaris*). *J. exp. Bot.* **11**, 81.

JACKSON, WM. T. (1959). The effects of pectinase and cellulase preparations on the growth and development of root hairs. *Physiol. Plant.* **12**, 502.

JANSEN, E. F., JANG, R., ALBERSHEIM, P. & BONNER, J. (1960). Pectic metabolism of growing cell walls. *Plant. Physiol.* **35**, 87.

JENSEN, H. L. (1942). Nitrogen fixation in leguminous plants. II. Is symbiotic nitrogen fixation influenced by Azotobacter? *Proc. Linn. Soc. N.S.W.* **67**, 205.

LJUNGGREN, H. (1961). *Ann. Rep. Rothamsted exp. Sta.* p. 85.

MELOH, K. A. (1961). *Untersuchungen zur Biologie und Bedeutung der endotrophen Mykorrhiza bei* Zea mays *L. und* Avena sativa *L.* Dissertation der Universität Köln.

MOSSE B. (1953). Fructifications associated with mycorrhizal strawberry roots. *Nature, Lond.* **171**, 974.

MOSSE, B. (1956). Fructifications of an *Endogone* sp. causing endotrophic mycorrhiza in fruit plants. *Ann. Bot., Lond.* **20**, 349.

MOSSE, B. (1957). Growth and chemical composition of mycorrhizal and non-mycorrhizal apples. *Nature, Lond.* **179**, 922.

MOSSE, B. (1959*a*). The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. *Trans. Brit. mycol. Soc.* **42**, 273.

MOSSE, B. (1959*b*). Experimental techniques for obtaining a pure inoculum of an *Endogone* sp., and some observations on the vesicular-arbuscular infections caused by it. *Proc. 9th int. bot. Congr.* **2**, 1728.

NUTMAN, P. S. (1949). Nuclear and cytoplasmic inheritance of resistance to infection by nodule bacteria in red clover. *Heredity*, **3**, 263.

PEUSS, H. (1958). Untersuchungen zur Ökologie und Bedeutung der Tabakmycorrhiza. *Arch. Mikrobiol.* **29**, 112.

SCHRADER, R. (1958). Untersuchungen zur Biologie der Erbsenmycorrhiza. *Arch. Mikrobiol.* **32**, 81.

TOMLINSON, J. A. & WEBB, M. J. W. (1960). Powdery mildew diseases. *Rep. nat. veg. Res. Sta. Warwick*, **10**, 43.

WOOD, R. K. S. (1955). Pectic enzymes secreted by pathogens and their role in plant infection. *Symp. Soc. gen. Microbiol.* **5**, 263.

WOOD, R. K. S. (1960). Pectic and cellulolytic enzymes in plant disease. *Annu. Rev. Plant Physiol.* **11**, 299.

EXPLANATION OF PLATES

PLATE I

Figs. 1–5. Vesicular-arbuscular infections in aseptically raised seedlings inoculated with sterile germinated spores of an *Endogone* sp. (L.S. root).

Fig. 1. Young infection, mainly arbuscular, in *Trifolium pratense*. Fungal penetration mediated by *Pseudomonas* sp. (isolate No. 1).

Fig. 2. Details of arbuscules in *Trifolium parviflorum*.

Fig. 3. Infection in *Dactylis glomerata*.

Fig. 4. Entry point and general infection in *T. parviflorum*. Fungal penetration mediated by sterile filtrate from dual culture of *T. parviflorum* and *Pseudomonas* sp. (isolate No. 1).

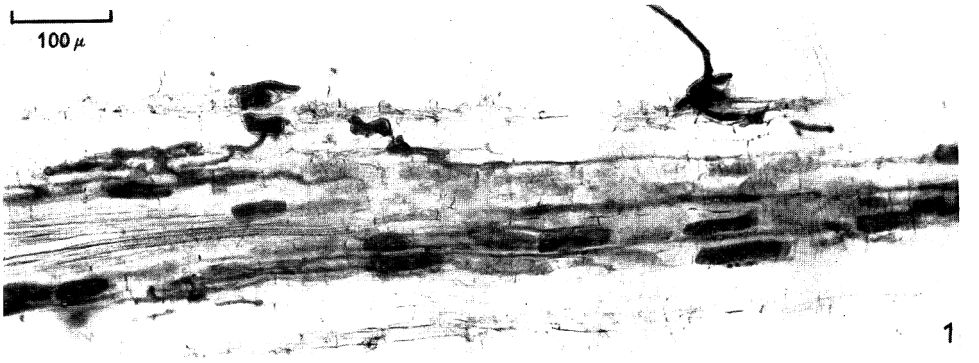
Fig. 5. Four-months-old infection in *T. parviflorum*. Prominent vesicles, absence of arbuscules and disintegrating cortex are typical of late infection stages when the seedling is nearly moribund.

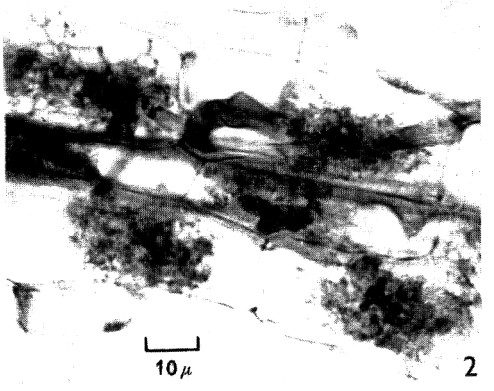
PLATE 2

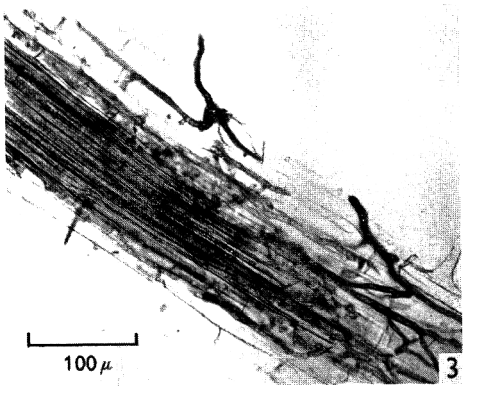
Fig. 6. Root of *T. parviflorum* with vigorous emerging hyphae which remained attached to the infected root when it was pulled out of the agar medium.

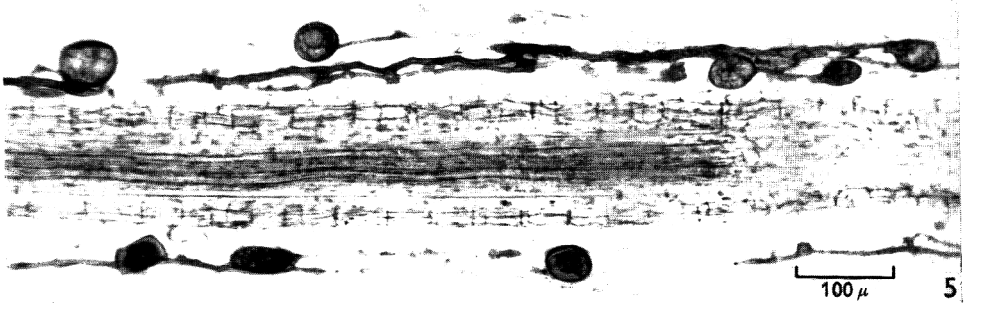
Fig. 7. Aseptic seedlings of *T. parviflorum* grown in Jensen's medium and inoculated with sterile Endogone spores. Mycorrhizal roots which are longer and more branched are marked *m* ——→ Fungal infection mediated by (*a*) Pectinol and (*b*) *Pseudomonas* sp. (isolate No. 1).

Fig. 8. Seedlings of *T. parviflorum* grown aseptically in Jensen's medium (*a*) without and (*b*) with an inoculum of *Pseudomonas* sp. (isolate No. 1).



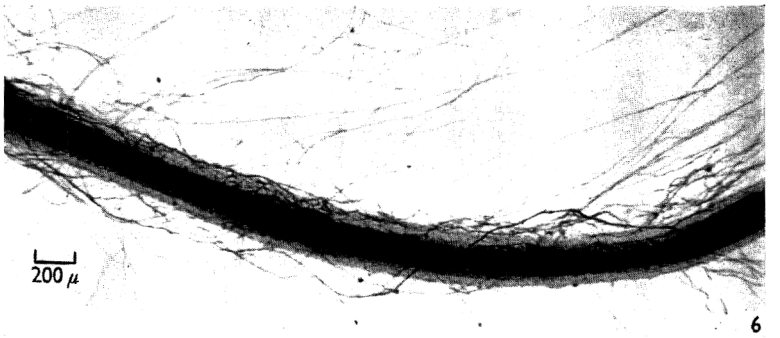




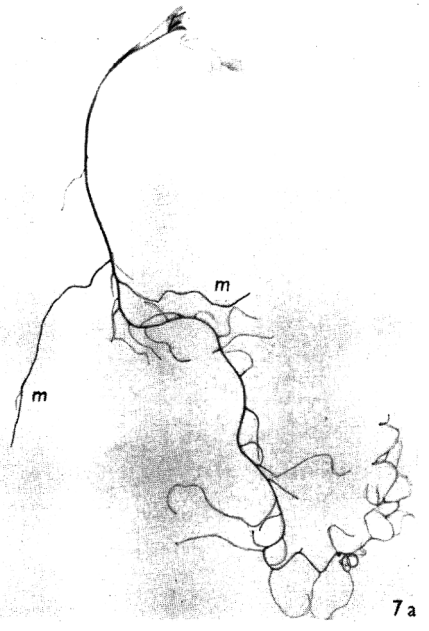


B. MOSSE

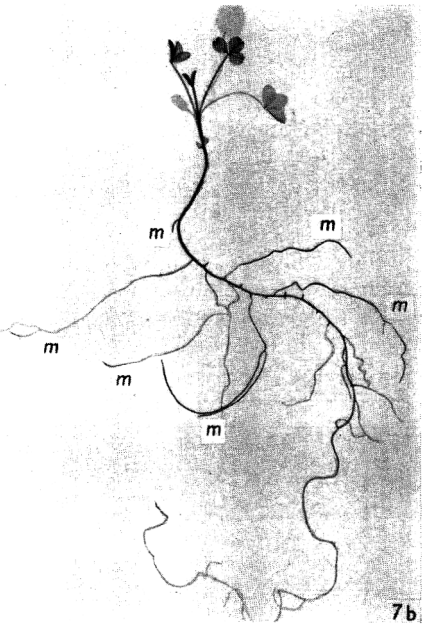
(*Facing p.* 520)



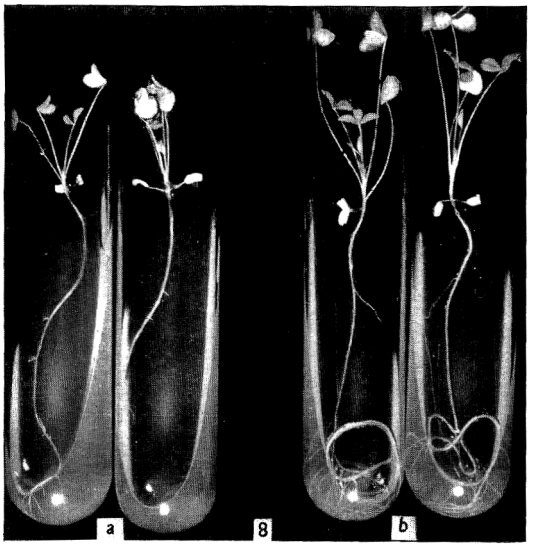
6



7a



7b



8

J. gen. Microbiol. (1962), **27**, 521–527
Printed in Great Britain

Effect of Chloramphenicol on Protein and Nucleic Acid Synthesis by *Shigella flexneri*

By R. B. YEE, SYLVIA PAN and H. M. GEZON
Graduate School of Public Health, University of Pittsburgh, Pittsburgh 13, *Pennsylvania, U.S.A.*

(*Received* 31 *July* 1961)

SUMMARY

Resting organisms of *Shigella flexneri* 3 were able to synthesize protein and nucleic acids in a chemically defined medium. Protein synthesis was markedly inhibited while ribonucleic acid (RNA) synthesis was stimulated by low concentrations of chloramphenicol; deoxyribonucleic acid synthesis was inhibited slightly. Neither glycine, L-phenylalanine nor L-tyrosine annulled the action of the antibiotic. Inhibition of protein synthesis and stimulation of RNA synthesis in resting organisms of a chloramphenicol-resistant mutant of *S. flexneri* 3 was observed only in the presence of high concentrations of chloramphenicol. The results presented in this paper show that chloramphenicol suppresses the growth of *S. flexneri* 3 by interfering with the synthesis of protein. The stimulation of RNA synthesis suggests that chloramphenicol may exert its inhibitory action by promoting the formation of RNA with altered biological activity.

INTRODUCTION

The exact biochemical nature of the mode of action of chloramphenicol on bacteria is not yet known. Investigations have shown the compound is a strong inhibitor of protein synthesis (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954; Pardee & Prestidge, 1956; Bernlohr & Webster, 1958). However, there is a lack of agreement about the effect of the drug on nucleic acid synthesis. Gale & Folkes (1953) showed that chloramphenicol stimulated nucleic acid production in *Staphylococcus aureus*. Wisseman *et al.* (1954) observed no effect in *Escherichia coli* strain B. Harrington (1958), on the other hand, reported that a slight enhancement of nucleic acid synthesis accompanied the chloramphenicol inhibition of protein synthesis in *E. coli* strain B. Pardee & Prestidge (1956) also with *E. coli* strain B found that low concentrations of the chloramphenicol did not suppress the production of nucleic acid, whereas high concentrations markedly inhibited the formation of deoxyribonucleic acid (DNA), and, to a lesser extent, ribonucleic acid (RNA). With *Azotobacter agilis*, Bernlohr & Webster (1958) showed that high concentrations of chloramphenicol markedly depressed protein, DNA and RNA synthesis while lower amounts inhibited only protein and DNA production.

Although chloramphenicol is of major importance in the therapy of shigellosis, studies on its effect on the metabolism of shigellas have not as yet been reported. An investigation has been begun to determine the mechanism of chloramphenicol action in a strain of *Shigella flexneri* 3. The effects of chloramphenicol on protein and in particular nucleic acid synthesis by this organism in a chemically defined medium has been studied and the results are presented in this paper.

METHODS

Cultures. Shigella flexneri 3 strain B-1003 was used. The origin of the organism and the maintenance of the reference stock culture were described previously (Pan, Yee & Gezon, 1957). The growth of this strain was inhibited by 2–4 μg. chloramphenicol/ml. as determined by the tube dilution method. The Szybalski gradient plate technique (Bryson & Szybalski, 1952) was used to isolate a chloramphenicol-resistant mutant of strain B-1003. Growth of the mutant was inhibited by 50–60 μg. chloramphenicol/ml.

Cultivation of organism. The organisms were cultured in a defined medium (DM) containing the following g./l. medium: 3·53 KH_2PO_4; 5·78 Na_2HPO_4; 5·00 NaCl; 0·24 $MgSO_4.7H_2O$; 2·50 D-glucose as an energy and carbon source; 2·50 $NH_4H_2PO_4$ as sole nitrogen source. The medium also contained 0·01 μg. nicotinamide/ml. This defined medium will be referred to as ammonium phosphate DM. For metabolic experiments, a loopful of growth from an 18 hr. ammonium phosphate DM slope culture was inoculated into the liquid DM. The liquid cultures were aerated continuously by vigorous shaking on a Burrell wrist action shaker. Incubation was carried out at 37° for 18 hr.

To cultivate the chloramphenicol-resistant mutant, the organisms were transferred from a stock semi-solid DM stab culture to an ammonium phosphate DM agar slope. After incubation for 18 hr. at 37°, a loopful of the slope culture was streaked on a Szybalski gradient plate containing chloramphenicol. The most resistant organisms on the gradient plate were then transferred to an ammonium phosphate DM agar slant containing 20 μg. chloramphenicol/ml. The resulting growth was resuspended in 2 ml. DM containing L-aspartic acid instead of $NH_4H_2PO_4$ as nitrogen source and inoculated into 400–500 ml. of the DM containing 20 μg. chloramphenicol/ml. Aspartic acid DM was used since resting organisms which had been grown in this medium exhibited higher degrees of protein and nucleic acid synthesis than ones grown in ammonium phosphate DM. Incubation of the culture was the same as that for the parent strain.

Preparation of organisms. After incubation, the organisms were harvested by centrifugation and washed three times with phosphate buffered saline (pH 6·9). For growth experiments, the organisms were used immediately; for resting cell experiments, they were aerated at 37° for 2–3 hr. by shaking in a Dubnoff metabolic shaking incubator, washed once, resuspended in saline, and stored overnight at 5°. The organisms were again aerated for 1 hr., centrifuged and washed once in buffered saline; these will be referred to as resting organisms. Multiplication in defined media during the period of the experiment was at a minimum.

Reaction system. Study of the effect of chloramphenicol on protein and nucleic acid synthesis in defined media was carried out with growing and with non-growing organisms. The organisms were suspended in a solution of KH_2PO_4, Na_2HPO_4, NaCl and nicotinamide; the concentration was adjusted so that the suspension gave 10 % light transmittance at 425 mμ with a light path of 18 mm. in a Coleman Model 6B spectrophotometer. This suspension was used in the experiments with non-growing organisms while in the experiments with growing organisms a 1/10 dilution of the suspension in the salt solution was used. Thirty ml. of suspension was mixed with 15 ml. of solution containing $NH_4H_2PO_4$ or a specified amino acid,

D-glucose and $MgSO_4.7H_2O$. The final concentration of all the compounds was the same as that in the defined media used for growth. The organisms were incubated at 37° in a Dubnoff metabolic shaking incubator; duplicate samples were removed for analysis. Nucleic acids and proteins were precipitated with cold 5% (w/v) trichloroacetic acid, sedimented by centrifugation at 2700 *g* for 40 min. at 0° and washed once with cold 5% (w/v) trichloroacetic acid. The precipitates for protein analysis were dissolved in N-NaOH. The precipitates for nucleic acid determinations were extracted by the method of Gale & Folkes (1953). For the estimation of ammonia utilization, the organisms were removed by centrifugation at 0° and the supernatant fluid analysed. Each experiment was replicated twice or more. The data presented in the figures are the results of single experiments.

Analytical methods. Ammonia was determined by nesslerization (Umbreit, Burris & Stauffer, 1957); protein by the method of Lowry, Rosebrough, Farr & Randall (1951); RNA by the Meijbaum technique (Colowick & Kaplan, 1957); DNA by the Burton modification of the Dische technique (Burton, 1956) and total nucleic acid by measuring the absorption of the extracts at 260 mμ. The Beckman model DU spectrophotometer was used in all the analyses. Numbers of viable organisms were determined by the plate count technique.

RESULTS

The results of experiments with growing cultures of *Shigella flexneri* 3 showed that multiplication, ammonia assimilation and protein DNA and RNA synthesis were suppressed by low concentrations of chloramphenicol. However, it could not be concluded from these findings that the inhibition of multiplication was the result of interference with the metabolic processes; the decrease in metabolic activity might merely reflect a decrease in multiplication. To test this hypothesis, non-multiplying or at least slowly multiplying preparations of organisms, designated resting organisms, were used. There was no detectable increase in numbers of viable organisms during the first 2 hr. of incubation in the defined media, although by this time maximum increases in protein and nucleic acid were attained (Fig. 1). Studies on ammonia assimilation by resting organisms in ammonium phosphate defined medium showed that the assimilation was not affected by chloramphenicol, of which concentrations as high as 100 μg./ml. were used. The previously observed inhibition of ammonia assimilation in growing cultures of *S. flexneri* 3 thus appears to be the result rather than the cause of the suppression of multiplication.

The ability of the resting organisms to synthesize protein and nucleic acids in the presence of various nitrogen sources in a mineral salts glucose base is shown in Fig. 1. The relatively simple nutritional requirements of *Shigella flexneri* was indicated by the results. The highest increases in protein and nucleic acids were obtained when the organisms were provided with either ammonium phosphate or L-aspartic acid. Although the degree of protein synthesis was the same in the presence of these two compounds, considerably more nucleic acids were produced when L-aspartic acid served as nitrogen source. Appreciably less protein synthesis and no detectable nucleic acid synthesis was observed with the other nitrogenous compounds.

The effect of low concentrations of chloramphenicol on protein and nucleic acid synthesis in aspartic acid DM is shown in Fig. 2; similar results were obtained with ammonium phosphate DM. The formation of protein was markedly depressed by chloramphenicol; there was almost complete inhibition with 2·0 μg./ml. In

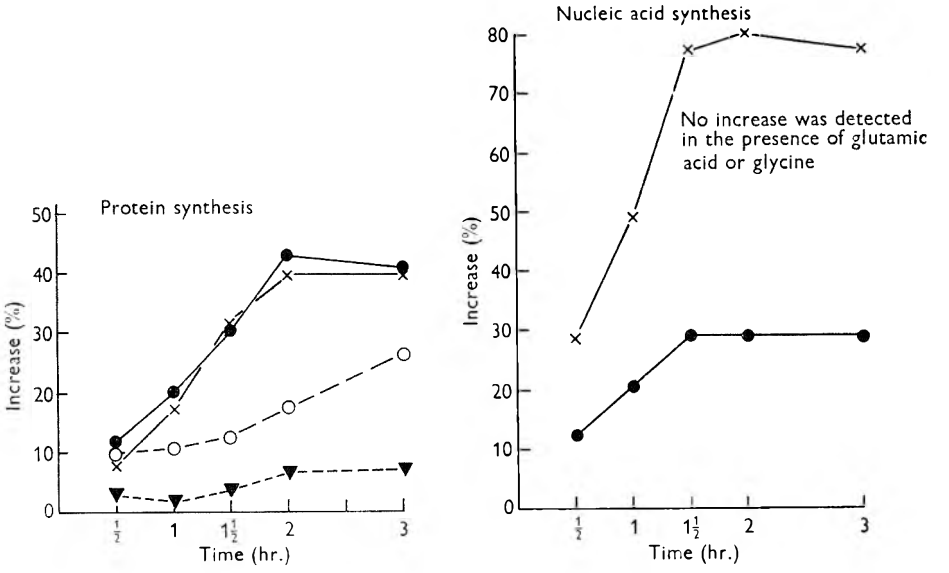


Fig. 1. Synthesis of protein and nucleic acid by resting organisms of *Shigella flexneri* 3 B-1003 in the presence of different nitrogen compounds: (●) $NH_4H_2PO_4$; (×) L-aspartic acid; (○) L-glutamic acid; (▼) glycine

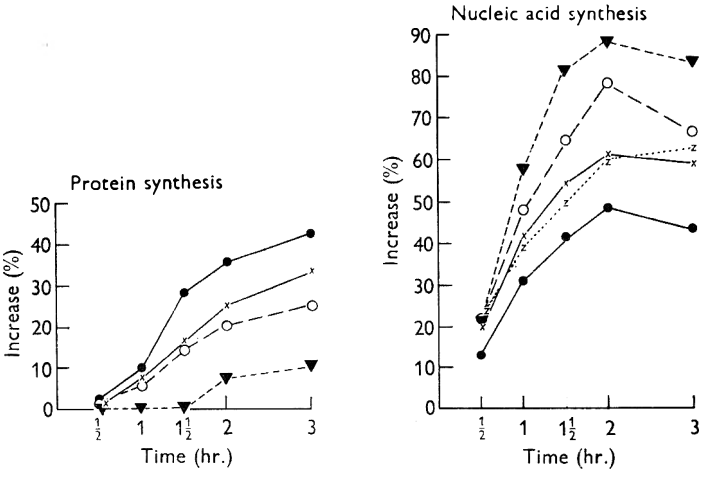


Fig. 2. Effect of chloramphenicol on protein and nucleic acid synthesis by resting *Shigella flexneri* 3 B-1003 in aspartic acid defined medium. Chloramphenicol concentrations (μg./ml.) used were: (●) 0; (×) 0·5; (○) 1; (▼) 2; (z) 10.

contrast to the observed action on protein production, nucleic acid synthesis was markedly stimulated by chloramphenicol; in the range of 0·5–2·0 μg. chloramphenicol/ml., the addition of increasing concentrations resulted in greater stimulation,

whereas beyond these concentrations the degree of stimulation was decreased. Analyses of the extracts by the Meijbaum technique showed that the observed increase was the result of stimulation of RNA synthesis. Low concentrations of chloramphenicol appeared to depress slightly the rate of DNA formation.

Harrington (1958) reported that the excretion of 260 mμ absorbing substances by *Escherichia coli* was increased considerably in the presence of 1·0 μg. chloramphenicol/ml. In contrast, concentrations of 0·5–2·0 μg./ml. had no effect on the rate of excretion of 260 mμ absorbing substances by resting *Shigella flexneri* 3 in aspartic acid DM. Various investigators (Mentzer, Meunier & Molho-LaCroix, 1950; Wooley, 1950; Foster & Pittillo, 1953) reported that glycine, L-phenylalanine or L-tyrosine annulled the growth inhibitory action exerted by chloramphenicol

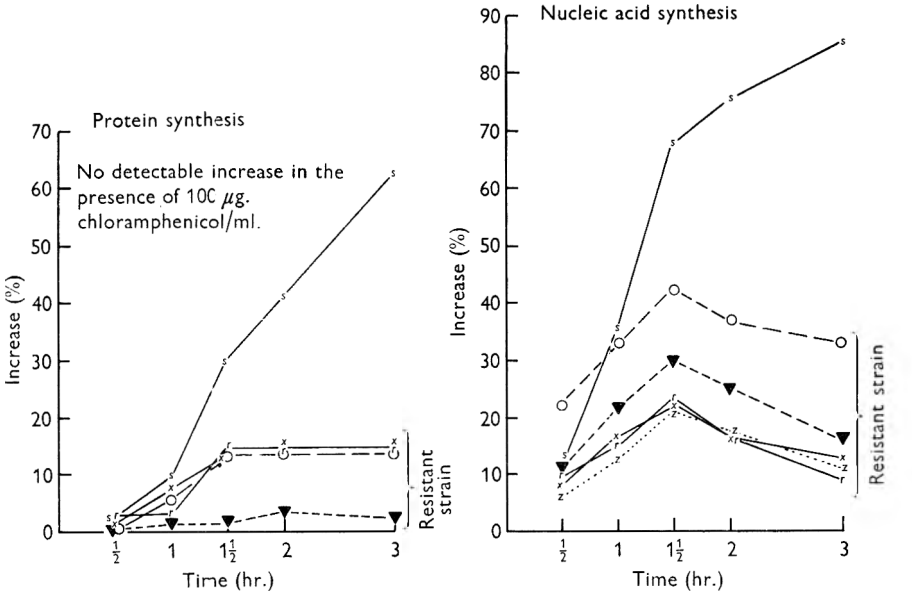


Fig. 3. Effect of chloramphenicol on protein and nucleic acid synthesis by resting organisms of a chloramphenicol-resistant mutant of *Shigella flexneri* 3 B-1003 in aspartic acid defined medium. Chloramphenicol concentrations (μg./ml.) used were: (*r*) 0; (×) 2; (○) 25; (▼) 50; (*z*) 100. The results with a chloramphenicol-sensitive strain (*s*) are included for comparison.

against *E. coli*. They suggested that the compound acts as an amino acid antagonist. Experiments were carried out to determine the effect of 0·125 % (w/v) of each of these amino acids on the action of chloramphenicol against protein and nucleic acid synthesis by resting *S. flexneri* 3 metabolizing in L-aspartic acid DM. Chloramphenicol concentrations of 1·0 μg. and 4·0 μg./ml. (causing about 50 % or complete inhibition of protein synthesis, respectively) were used. None of these amino acids demonstrated an antagonistic action against the effects of chloramphenicol. These results are similar to those reported by Hopps *et al.* (1956). Chloramphenicol does not appear to suppress protein synthesis by acting as an amino acid antagonist.

Protein synthesis by chloramphenicol-resistant mutants would be expected to be insensitive to low concentrations of the antibiotic if the synthesis were the

site of chloramphenicol inhibition. With this in mind, experiments with resting organisms of a resistant mutant of *Shigella flexneri* strain B-1003 were performed. The results are shown in Fig. 3; 2 μg. chloramphenicol/ml. which markedly inhibited protein synthesis and stimulated RNA production by the wild type had no effect on the resistant mutant. A concentration of 50 μg. chloramphenicol/ml., which approaches that required for growth inhibition of the mutant, was necessary for the depression of protein synthesis and the stimulation of RNA synthesis. The results in Fig. 3 also show that the synthesizing activity of the resistant organisms was considerably lower than that of the wild type. This finding was not unexpected since it had been observed that cultures of the resistant mutant grew slower than those of the wild type.

DISCUSSION

The findings presented here suggest that, as in *Staphylococcus aureus* and *Escherichia coli*, chloramphenicol suppresses the multiplication of *Shigella flexneri* 3 B-1003 through interference with protein synthesis. Further support for this conclusion is provided by the results of experiments with the chloramphenicol-resistant mutant. The increased resistance of multiplication to chloramphenicol was accompanied by a similar increase in the resistance of protein synthesis by the mutant. The observed stimulation of RNA synthesis by chloramphenicol in *S. flexneri* 3 B-1003 is in agreement with the findings of Gale & Folkes (1953) with *S. aureus* but not with those of Wisseman *et al.* (1954) who stated they were unable to demonstrate stimulation in *E. coli*. Thus the chloramphenicol may differ in its action with different species. Another possible explanation for the lack of agreement may be the use of non-multiplying organism suspensions in the present study and that of Gale & Folkes and the use of rapidly growing organisms by Wisseman *et al.* This was suggested by Harrington (1958).

The stimulation of RNA synthesis in resting *Shigella flexneri* 3 B-1003 suggests that chloramphenicol may suppress the formation of protein by altering synthesis of the nucleic acid. As a result, RNA which cannot participate in protein synthesis may be produced or, as proposed by Gale (1958) and Ramsey (1958), the drug may change or interfere with the function of RNA. Pardee, Paigen & Prestidge (1957), Niedhardt & Gros (1957) and Nomura & Watson (1959) presented evidence which suggests that RNA formed by *Escherichia coli* in the presence of chloramphenicol is different from that produced in the absence of the compound. Also, Horiuchi, Horiuchi & Mizuno (1959) reported that RNA synthesized by *E. coli* in the presence of chloramphenicol did not participate in the formation of protein in intact organisms.

This investigation was carried out under the sponsorship of the Commission on Enteric Infections, Armed Forces Epidemiological Board, and financially supported by the Surgeons General, Department of the Army and Department of the Navy. We are grateful to Parke, Davis and Co. for a generous supply of chloramphenicol. We also wish to thank Mr F. Erdlejac and Mr J. McElligott for their technical assistance.

REFERENCES

BERNLOHR, R. W. & WEBSTER, G. C. (1958). Effect of chloramphenicol on protein and nucleic acid metabolism in *Azotobacter agilis*. *J. Bact.* **76**, 233.

BRYSON, V. & SZYBALSKI, W. (1952). Microbial selection. *Science*, **116**, 45.

BURTON, K. (1956). A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315.

COLOWICK, S. P. & KAPLAN, N. O. (1957). *Methods in Enzymology*, vol. 3. New York: Academic Press Inc.

FOSTER, J. W. & PITTILLO, R. F. (1953). Metabolite reversal of antibiotic inhibition, especially reversal of aureomycin inhibition by riboflavin. *J. Bact.* **66**, 478.

GALE, E. F. & FOLKES, J. P. (1953). The assimilation of amino acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochem. J.* **53**, 493.

GALE, E. F. (1958). Specific inhibitors of protein synthesis. In *The Strategy of Chemotherapy. Symp. Soc. gen. Microbiol.* **8**, 212.

HARRINGTON, M. G. (1958). The action of chloramphenicol on protein and nucleic acid synthesis by *Escherichia coli* strain B. *J. gen. Microbiol.* **18**, 767.

HOPPS, H. E., WISSEMAN, C. L., JR., HAHN, F. E., SMADEL, J. E. & HO, R. (1956). Mode of action of chloramphenicol. 4. Failure of selected natural metabolites to reverse antibiotic action. *J. Bact.* **72**, 561.

HORIUCHI, T., HORIUCHI, S. & MIZUNO, D. (1959). Non-participation in protein synthesis of the RNA synthesized in the presence of chloramphenicol in *Escherichia coli*. *J. med. Sci.* **12**, 99.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MENTZER, C., MEUNIER, P. & MOLHO-LACROIX, L. (1950). Faits de synergie et d'antagonisme entre la chloromycétine et divers amino-acids vis-à-vis de cultures d' *E. coli*. *C.R. Acad. Sci., Paris*, **230**, 241.

NIEDHARDT, F. C. & GROS, F. (1957). Metabolic instability of the ribonucleic acid synthesized by *Escherichia coli* in the presence of chloromycetin. *Biochim. biophys. Acta*, **25**, 513.

NOMURA, M. & WATSON, J. D. (1959). Ribonucleoprotein particles with chloromycetin inhibited *Escherichia coli*. *J. mol. Biol.* **1**, 204.

PAN, S. F., YEE, R. & GEZON, H. M. (1957). Studies on the metabolism of Shigella. I. The occurrence of a tricarboxylic acid cycle in *Shigella flexneri*. *J. Bact.* **73**, 402.

PARDEE, A. B. & PRESTIDGE, L. S. (1956). The dependence of nucleic acid synthesis on the presence of amino acids in *Escherichia coli*. *J. Bact.* **71**, 677.

PARDEE, A. B., PAIGEN, K. & PRESTIDGE, L. (1957). A study of the ribonucleic acid of normal and chloromycetin-inhibited bacteria by zone electrophoresis. *Biochim. biophys. Acta*, **23**, 162.

RAMSEY, H. H. (1958). Protein synthesis as a basis for chloramphenicol-resistance in *Staphylococcus aureus*. *Nature, Lond.* **182**, 602.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. H. (1957). *Manometric Techniques*. Minneapolis: Burgess Publishing Co.

WISSEMAN, C. L., JR., SMADEL, J. E., HAHN, F. E. & HOPPS, H. E. (1954). Mode of action of chloramphenicol. 1. Action of chloramphenicol on assimilation of ammonia and on synthesis of proteins and nucleic acids in *Escherichia coli*. *J. Bact.* **67**, 662.

WOOLLEY, D. W. (1950). A study of non-competitive antagonism with chloromycetin and related analogues of phenylalanine. *J. biol. Chem.* **185**, 293.

J. gen. Microbiol. (1962), 27, 529
Printed in Great Britain

Books Received

Human Genetics, British Medical Bulletin. 17, no. 2. September 1961. Published by the Medical Department, The British Council, 65 Davies Street, London. Price 20*s*.

Virus and Rickettsial Diseases of Man. By S. P. BEDSON, A. W. DOWNIE, F. O. MACCALLUM and C. H. STUART-HARRIS. London: Edward Arnold (Publishers) Ltd. July 1961. 461 pp. Price 60*s*.

Euglena, an Experimental Organism for Biochemical and Biophysical Studies. By J. J. WOLKEN. June 1961. Distributed by The Rutgers University Press, New Brunswick, New Jersey, U.S.A. Price $4.50.

Précis de Bactériologie. By R. LE GUYON. Published by Doin et Cie, 8 Place de l'Odéon, Paris 6e. 955 pp.

Pioneer Microbiologists of America. By P. F. CLARK. 1961. The University of Wisconsin Press, Wisconsin, U.S.A. 369 pp. Price $6.00.

Annual Review of Microbiology. 1961. Ed. by C. E. CLIFTON, S. RAFFELL and M. P. STARR. Published by Annual Reviews, Inc., Palo Alto, California, U.S.A. 349 pp. Price $7.00.

Industrial Microbiology. By A. H. ROSE. 1961. Published by Butterworth and Co. (Publishers) Ltd., 4 and 5 Bell Yard, London. 286 pp. Price 60*s*.

Biological Stains. By H. J. CONN. Published by Baillière, Tindall and Cox, 7 and 8 Henrietta Street, London. 1961. 355 pp. Price 72*s*.

Index of Authors

Index of Subjects